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MALARIA DIAGNOSIS IN CHILDREN: ACCURACY AND QUALITY OF RAPID DIAGNOSTIC TESTS

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Voor papa

Leo Cornelis Maltha (10/11/1952 – 12/11/2013)

Wat ik niet zeggen kan
en niet kan schrijven
zal ergens diep in mij
toch bij me blijven

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List of abbreviations

ACT	Artemisinin-based combination therapy
CE	Conformité Européenne
CFR	Case-fatality rate
CI	Confidence interval
CLSI	Clinical Laboratory standard institute
DCS	Decreased Ciprofloxacin Susceptibility
DNA	Deoxyribonucleic acid
EC	European Community
EC-REP	European authorized representative
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EQA	External quality assessment
ESBL	Extended-spectrum beta-lactamases
EUCAST	European committee on antimicrobial susceptibility testing
FDA	Food and drug administration
FIND	Foundation for innovative new diagnostics
G6PD	Glucose-6-phosphate dehydrogenase
GMP	Good manufacturing practice
Hb	Hemoglobin
HIV	Human Immunodeficiency Virus
HRP-2	Histidine-rich protein 2
IFU	Instructions for use
IQR	Interquartile range
IRB	Institutional review board
ISO	International organization for standardization
ITM	Institute of Tropical Medicine
IVD	In vitro diagnostic medical device
K	Kappa value
MDR	Multidrug resistance
MIC	Minimal inhibitory concentration
N	Number
NA	Not applicable

NTS	Non-Typhoid *Salmonella*
P.	*Plasmodium*
pan-pLDH	pan *Plasmodium* parasite lactate dehydrogenase
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PfHRP2	*Plasmodium falciparum*-specific histidine-rich protein-2 (see also HRP-2)
PfHRP3	*Plasmodium falciparum*-specific histidine-rich protein-3
Pf-pLDH	*Plasmodium falciparum*-specific parasite lactate dehydrogenase
pLDH	parasite lactate dehydrogenase
Pm	*Plasmodium malariae*
Po	*Plasmodium ovale*
Pv	*Plasmodium vivax*
Pv-pLDH	*Plasmodium vivax*-specific parasite lactate dehydrogenase
QC	Quality control
RBC	Red blood cell
RDT(s)	Rapid diagnostic test(s)
SBET	Stand-by emergency treatment
STARD	Standards for reporting of diagnostic accuracy
WBC	White blood cell
WHO	World Health Organization.

Chapter 1. Introduction

Parts of this introduction have been adapted from

- **Maltha J,** Jacobs J. Clinical practice: The diagnosis of imported malaria in children *Eur J Pediatr* 2011, 170: 821-829.
- **Maltha J,** Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. *Clin Microbiol Infect* 2013, 19: 399-407.
- **Maltha J,** Gillet P, Jacobs J. Malaria rapid diagnostic tests in travel medicine. *Clin Microbiol Infect* 2013, 19: 408-415.

Burden of malaria

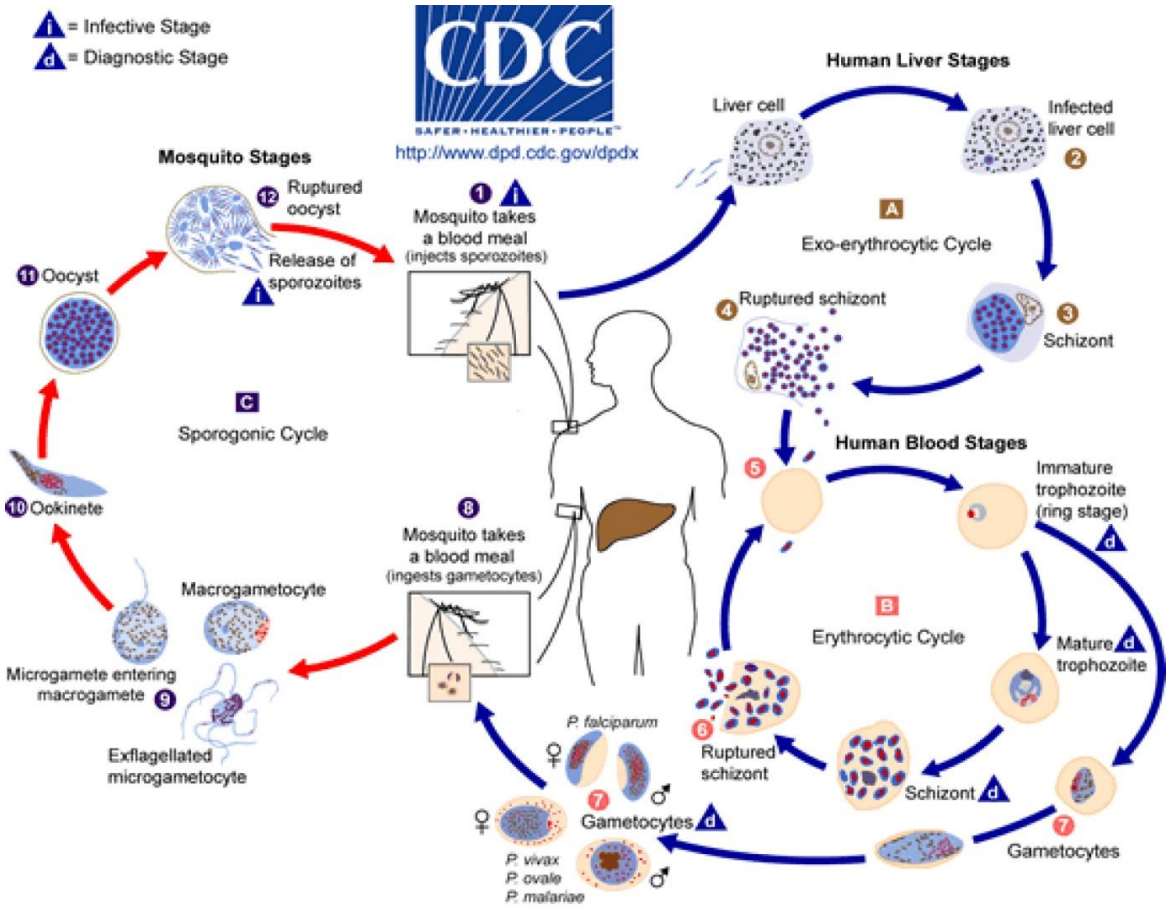
Approximately half of the world's population is at risk of acquiring malaria [1]. An estimated 219 million persons get infected annually and 600,000 people die [1]. Children are mostly affected: 86% of deaths occurs in children <5 years of age, most of them in Sub-Saharan Africa. Other vulnerable groups are pregnant women and non-immune travelers.

What is malaria? [2]

The parasite

Malaria is caused by a parasite called *Plasmodium*, which is transmitted through the bites of the infected *Anopheles* mosquito. There are four human *Plasmodium* species: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale and Plasmodium malariae.* Recently, human infections with a fifth species, *Plasmodium knowlesi*, which is usually found in monkeys, have been reported (48). Figure 1 depicts the life cycle of malaria parasites in humans.

Figure 1. *Plasmodium* life cycle



The Plasmodium life cycle in humans (right, blue arrows) and mosquitos (left, red arrows).
Retrieved from Centers for Disease Control and Prevention (CDC). Available at http://www.cdc.gov/malaria/about/biology/

The cycle in the human host: from liver to blood, hypnozoites, relapse and recrudescence

Plasmodium has a sexual cycle inside the mosquito and an asexual cycle inside man. During the bite of an infected female mosquito, *Plasmodium* sporozoites are injected in the dermis, they migrate into the blood and move to liver cells where they develop into liver schizonts. After the rupture of

the schizonts (approximately one week after infection), merozoites are released into the bloodstream and each of them will invade a red blood cell (RBC). There they develop into ring forms (trophozoites) which mature into schizonts that divide into merozoites which invade new RBCs. The rupture of the schizont and release of merozoites give rise to the so-called malaria paroxysms with spiking fever and rigors. The time required to complete the RBC cycle (from invading merozoites over trophozoites to rupturing schizont) depends on the species: for *P. falciparum*, *P. vivax* and *P. ovale*, the cycle takes 48 hours, for *P. malariae* and *P. knowlesi* it takes 72 hours and 24 hours respectively. In non-immune patients however, particularly when infected with *P. falciparum*, parasites tend to mature asynchronously, resulting in irregular fever patterns [3].

After one or two weeks, gametocytes, the sexual form of *Plasmodium*, will be produced. These gametocytes continue the sexual cycle when taken up by a mosquito during a next blood meal. The sexual cycle will be completed over a period of two weeks, where after the mosquito can infect another human host.

In case of *P. vivax* and *P. ovale* infections, dormant liver stages (hypnozoites) may cause infections (so-called relapses) weeks to months after adequate prophylaxis or effective blood-stage therapy of a primary infection. *P. malariae* may persist in the blood at undetectable levels without causing symptoms for many years and may subsequently develop into clinical illness in patients under immunosuppressive drugs or after splenectomy (also called recrudescence) [3]. Although rare, recrudescence has been reported for *P. falciparum* as well [4–7].

Geographic distribution of Plasmodium species

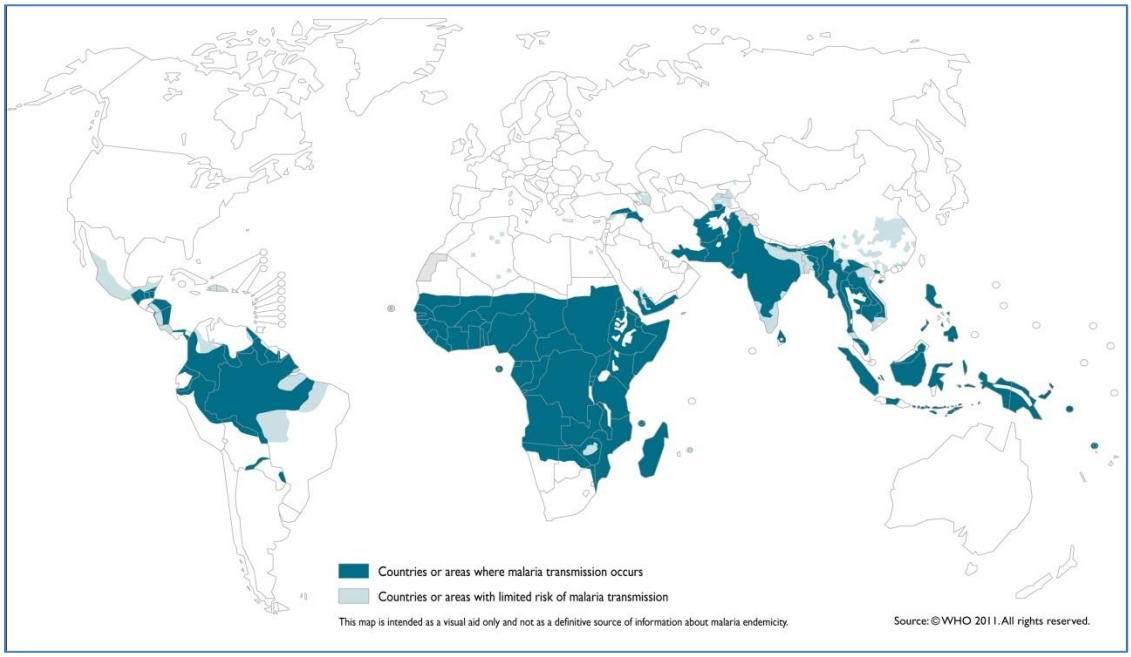
Figure 2 displays the countries and areas where malaria transmission occurs. *P. falciparum* is the predominant species in sub-Saharan Africa, where *P. vivax* is virtually absent, except for some areas in Eastern Africa, as most black Africans lack the Duffy blood group antigen which enables *P. vivax* to enter the RBC. *P. ovale* is mainly restricted to Africa, New Guinea and the eastern parts of Indonesia and the Philippines, but rare cases have been observed in the Middle East and Asia [8]. *P. malariae* is the second most common species in Africa and is also distributed in other geographic areas [3,8]. South-America is predominated by *P. vivax* though *P. falciparum* is present as well. The same goes for the Indian sub-continent and Southeast Asia where *P. malariae* is also prevalent. Most *Plasmodium knowlesi* infections have been reported from Malaysian Borneo [9], but have also been observed in other Southeast Asian countries [10,11].

Clinical symptoms

Children with uncomplicated malaria may present with symptoms mimicking other diseases. Fever is common, is often intermittent and may be absent upon presentation. Other symptoms may include vomiting, headaches, chills, muscle aches and anorexia, abdominal pain and diarrhea and in the case of *Plasmodium vivax* intense rigors may occur [12]. The clinical picture may be indistinguishable from many other common childhood infections like gastroenteritis (vomiting + diarrhea), respiratory infection or influenzae.

In the case of *P. falciparum,* severe malaria may develop rapidly if no adequate treatment is started. Symptoms and signs of severe malaria include severe anemia, convulsions, coma, respiratory distress and shock [13], symptoms that may also be present in other life-threatening diseases like bacterial sepsis or meningitis [12,14]. *P. vivax* and *P. knowlesi* can also cause severe malaria though to a lesser extent [15,16].

Figure 2. Countries or areas at risk of malaria transmission



Retrieved from WHO. Malaria, countries or areas at risk of transmission, 2010.

Treatment of malaria [17]

Artemisinin-based combination therapy (ACT) is recommended for uncomplicated *falciparum* malaria. For severe *P. falciparum* malaria intravenous artesunate is recommended, alternatively intravenous quinine can be given. *P. vivax* should be treated with chloroquine, or when resistance has been observed, with an ACT. In addition, primaquine should be administered to eradicate the liver hypnozoites. *P. ovale* should be treated with chloroquine and primaquine. *P. malariae* can be treated with chloroquine alone.

Parasite based diagnosis

In the past, patients in endemic countries were treated based on clinical symptoms alone. In view of the emergent resistance of *P. falciparum* to choloroquine and the need for more expensive artemisinin-based combination therapy (ACT), the World Health Organization (WHO) recommends treatment only after parasite based confirmation of the diagnosis, *i.e.* demonstration and identification of the parasite [17]. Microscopy or malaria rapid diagnostic tests (RDTs) can be used. Microscopy has the advantage of species identification and determination of parasite density. However, as microscopy is often unavailable in many malaria endemic settings or of low quality [18–21], malaria rapid diagnostic tests (RDTs) are recommended as the diagnostic method of choice [17]. In 2006, in high endemic areas it was still recommended to treat children under 5 presumptively as malaria was thought to be the most common cause of illness. More recent studies have however shown that a decreasing proportion of children presenting with fever are ill due to malaria [22], and that parasite-based treatment in children is safe [23]. Therefore, since March 2010, WHO extended the recommendation for the parasite-based treatment to children below five years old [17].

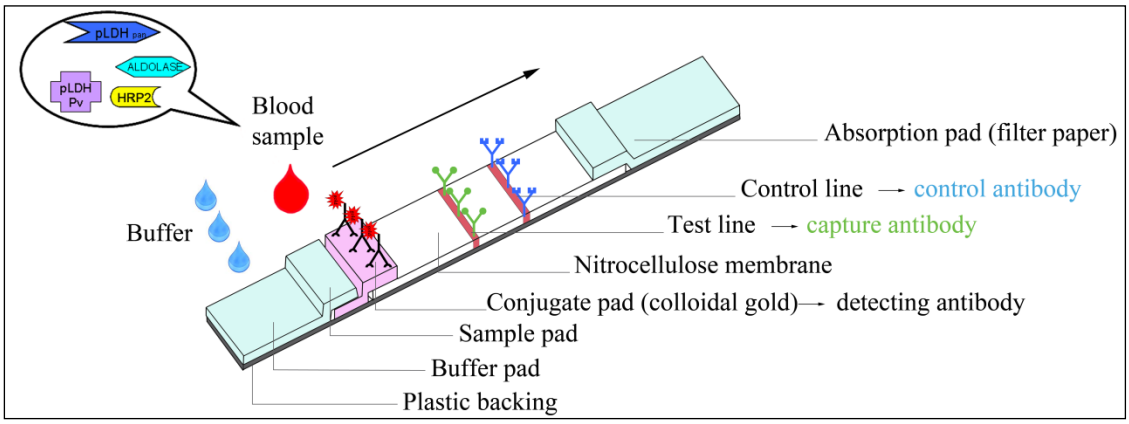
Malaria rapid diagnostic tests

Mechanisms of action

RDTs are handheld devices in which malaria is visualized by a colored line on a nitrocellulose strip (Figure 3).The nitrocellulose strip is mostly embedded in a plastic cassette; occasionally, this strip

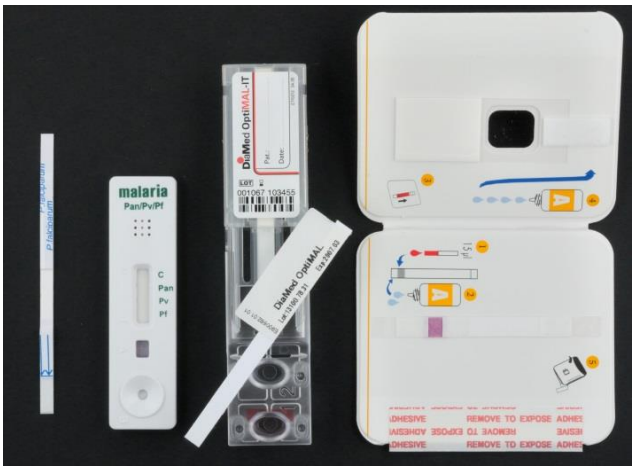
may present as a dipstick (self-standing strip to be dipped in a tube) or be enclosed in a cardboard format (Figure 4). When applying only 5 microliter of human blood, malaria can be detected by an antigen-antibody reaction on the test strip. The mechanism of action is explained in Figure 3.

Figure 3. Schematic drawing of a malaria rapid diagnostic test



Sequence of events when performing a malaria RDT. Blood and buffer are applied to the sample and buffer pad respectively. They are attracted by the capillary action of the absorption pad and start to migrate. First, they pass the conjugate pad, which contains a detection antibody targeting a Plasmodium antigen, such as PfHRP2, Pf-pLDH, Pv-pLDH, pan-pLDH or aldolase (for abbreviations see text). This detection antibody is a mouse-antibody that is conjugated to a signal, mostly colloidal gold. If present in the sample, the Plasmodium antigen is bound to this detection antibody-conjugate. Next, the antigen-antibody-conjugate complex migrates further until it is bound to the capture antibody, which binds to another site of the Plasmodium target antigen. As the capture antibody is applied on a narrow section of the strip, the complex with the conjugated signal will be concentrated and by virtue of the colloidal gold will become visible as a colored line. The excess of detection antibody-conjugate that was not bound by the antigen and the capture antibody moves further until it is bound to a goat-raised anti-mouse antibody, thereby generating a control line [2].

Figure 4. Different platforms of RDTs



From right to left: dipstick, cassette, hybrid and cardboard. [32]

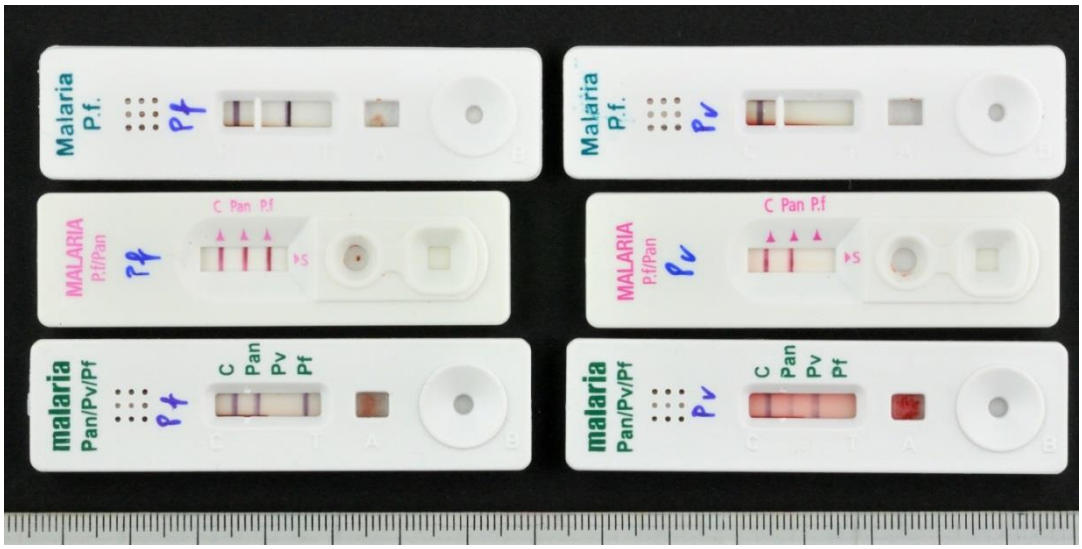
Plasmodium antigens

The following antigens may be detected by RDTs: *P. falciparum*-specific histidine-rich protein-2 (*Pf*HRP2), parasite lactate dehydrogenase (pLDH) which can be specific to *P. falciparum* (Pf-pLDH), *P. vivax* (Pv-pLDH) or common to all *Plasmodium* species (pan-pLDH) or aldolase.

The first antigen used for development of RDTs was *Pf*HRP2, a protein produced by asexual forms and young gametocytes of *P. falciparum* [24,25]. It is expressed on the RBC membrane and readily

diffuses into plasma [26]. Due to its slow clearance it may persist in the blood for more than 5 weeks after effective treatment of infection [27–30], which leads to low specificity in high endemic areas.
The enzyme pLDH is used in the glycolytic pathway of all *Plasmodium* species and is produced by both sexual and asexual forms [31]. Aldolase is another enzyme of the glycolytic pathway of the malaria parasite, it is common to all *Plasmodium* species and has also been incorporated as detection antigen in some of the commercial available RDT products.
Malaria RDTs are available in different formats using different combinations of detection antigens. Two-band RDTs consist of a control line and one test line. Three-band RDTs contain two test lines, usually targeting *P. falciparum* (*Pf*HRP2 or Pf-pLDH) and a pan-malaria antigen (pan-pLDH or aldolase). Four-band RDTs detect *P. falciparum*, a pan-malaria antigen and Pv-pLDH (Figure 5).

Figure 5. Two-, three- and four-band malaria RDTs (above, middle and below respectively) run with a *P. falciparum* sample (left) and a *P. vivax* sample (right).



The two-band RDT shows the control line and a *P. falciparum* (*Pf*HRP2)-line for the *P. falciparum* sample and only a control line for the *P. vivax* sample: correct reporting is "*P. falciparum*" and "no *P. falciparum* detected" respectively. The three-band RDT shows - apart from the control line - *Pf*HRP2 and pan-pLDH test lines for *P. falciparum*: correct reporting is "*P. falciparum*, mixed infection with non-*falciparum* species not excluded". The *P. vivax* sample shows only a pan-pLDH test line; correct reporting is "non-*falciparum* species". For the *P. falciparum* sample, the four-band RDT shows test lines for the pan-pLDH and *Pf*HRP2 test lines: correct reporting is "*P. falciparum*, mixed infection with *P. ovale/P. malariae* not excluded". For the *P. vivax* sample, pan-pLDH and Pv-pLDH test lines are visible; correct reporting is "*P. vivax*, mixed infection with *P. ovale/P. malariae* not excluded".

P. falciparum diagnosis: PfHRP2 versus Pf-pLDH

Most commercially available RDTs detect *Pf*HRP2 for the diagnosis of *P. falciparum.* Reported advantages of *Pf*HRP2 over Pf-pLDH-detection are higher sensitivity at low parasite density (< 100/microliter) [33] and lower susceptibility to environmental conditions like heat and humidity [34]. Pf-pLDH may however have other advantages, such as not being affected by the prozone effect, *i.e.* test lines of low intensity or absent test lines at high parasite densities [35]. Furthermore, no sequence variation of the encoding gene has been observed [36,37] in contrast to *Pf*HRP2 [38–40]. However, the impact of *Pf*HRP2 sequence variation on RDT accuracy remains under debate [32,38]. Finally, *Pf*-pLDH-detecting RDTs may perform better in areas where *pfhrp2* gene deletions have been observed [41].

Use and regulation of RDTs

From less than 200,000 RDTs used worldwide in 2005, this has rapidly expanded to 88,000,000 in 2010 and an annual need of 1.5 billion RDTs has been forecasted (Roll Back Malaria, http://www.rollbackmalaria.org/psm/index.html). Currently more than 200 products from 60 manufacturers are available [42]. Substandard RDTs are widespread in resource-limited settings [43,44] and lot-to-lot variations may affect performance of RDTs [45]. WHO provides quality control by inspection of manufacturing sites, systematic assessment of RDT products and their performance, a region based lot control program and provision of job aids and training material. There are however no controls at the bench and, except for a few brands (http://www.finddiagnostics.org/programs/malaria-afs/malaria/rdt-job-aids/), the provided job aids are generic whereas the RDT products on site may have different design and procedures.

RDTs for the diagnosis of severe malaria

In 2011, 87 out of 99 countries with on-going malaria transmission had adopted the policy of universal testing of malaria before start of treatment [1]. Several studies have shown safety of this strategy in uncomplicated malaria [23,46,47] although others debate this [48,49]. For children with signs of severe malaria this strategy has however not been extensively evaluated. In many remote settings, children may be diagnosed with malaria at health center level (provided RDTs are available) without further referral because of economic and geographic accessibility. As except for RDTs no other diagnostic method is available at the health center, further exploration to alternative causes of illness are limited and treatment decisions are based on clinical suspicion and malaria diagnosis by RDT. At the hospital level, malaria RDTs may be used rather than microscopy, because of lower workload and faster diagnosis, particularly at night. The RDT product most appropriate in this setting should be identified (*Pf*HRP2 versus Pf-pLDH), high sensitivity is needed but also high specificity as other life-threatening diseases may be missed. *Pf*HRP2-detecting RDTs are reported to have high sensitivity, also at very low parasite densities, but low specificity in high endemic settings in contrast to Pf-pLDH-detecting RDTs [33]. To refine the diagnostic algorithm in children with severe illness it is important to know how often malaria and other life-threatening diseases with similar symptoms like bacteremia and meningitis occur.

Malaria in travelers

Yearly more than 125 million travelers visit a malaria endemic country [50]. Approximately 10,000 cases of imported malaria are reported annually, *i.e.* infection acquired in endemic setting but symptoms appearing after return to home country, but this may be as high as 30,000 [50]. Children account for 10-15% of all imported malaria cases [2]. Travelers or expats who stay abroad for a longer time may fall ill during their stay abroad. As malaria diagnosis is not always within reach or of low quality and counterfeit medications are not uncommon, the use of standby emergency treatment is increasingly recommended [51], and would ideally be preceded by a high quality self-diagnostic test. This idea has been abandoned 10-15 years ago as several studies showed poor performance of RDTs in the hands of sick travelers [52–55]. Since then, RDTs have undergone improvements and self-use by travelers may be reconsidered. RDTs are now widely available through the internet without need of consultation or prescription. Internet-sold RDTs are however of unknown quality and there is currently no regulation of RDT products sold online [43].

Study objectives

Although malaria RDTs have been studied extensively, during evaluations of malaria RDTs and visits to field settings we observed shortcomings in the accuracy of RDTs, at the level of end-user performance as well as to their use in the diagnostic algorithm. The shortcomings observed have led to research questions that have been addressed in this thesis.

P. falciparum samples generating false positive P. vivax specific test lines (Chapter 2)
First of all, we observed anecdotal false positive Pv-pLDH lines in case of *P. falciparum* samples with high parasite densities. We assessed the degree to which this occurs and addressed the question whether all Pv-pLDH RDT products are affected in Chapter 2.

Which RDT for the Peruvian Amazon? (Chapter 3)
Colleagues in Peru recently described existence of *pfhrp2* gene deletions, the gene encoding the protein *Pf*HRP2 which is detected by most malaria RDTs, in *P. falciparum* field isolates. This observation incited us to do an extensive evaluation of different RDT products in the Peruvian Amazon, where most malaria cases occur. RDTs – if chosen appropriately - could highly contribute to malaria case management in this area where the majority of people at risk of acquiring malaria have no access to health facilities with a microscope.

End-user friendliness of RDTs (Chapter 4)
Accurate instructions for use (IFU) are crucial for RDT performance and interpretation. During field visits we occasionally observed shortcomings in RDT IFUs and content. Moreover, a study on the external quality assessment of malaria RDTs revealed that some of the errors in interpretation were embedded in the IFUs supplied with the RDT. Therefore we assessed a large panel of RDT products for correctness and completeness of their IFU, the quality of packaging and labeling of the RDT box, device and buffer vial.

RDTs for self-use by travelers? (Chapter 5)

> *MALARIA SELF-TEST?* *Source: www.lonelyplanet.com*
>
> *Hi,*
>
> *I'm going to be spending 3-4months cycling through Kenya, Tanzania and Mozambique solo.*
>
> *My travel clinic doctor has suggested i might want to take a malaria self-test kit with me as well as some treatment medication in case i find myself with malaria symptoms away from a clinic with the necessary medication. The latter sounds like a good idea, but is the test kit really worth it?*

Malaria RDTs are now freely available through the internet and may be of interest to expats and long-term travelers. There is no strict regulation of RDT sale through internet and while the RDT products promise excellent performance, diagnostic accuracy as well as quality of instructions of these products are not known. We addressed this in Chapter 5.

RDTs for children suspected of severe malaria (Chapters 6 and 7)

In malaria endemic areas, children with severe febrile illness are often diagnosed as having severe malaria. Bacterial infections are however not uncommon and co-infections with malaria may occur. In Burkina Faso there are hardly any data about the prevalence of bacterial infections. In Chapter 6 we assessed the proportions of severe malaria versus bacteremia in children presenting with severe febrile illness as well as the frequency of co-infections. In Chapter 7 we compare the diagnostic accuracy of *Pf*HRP2 versus Pf-pLDH-detection in children presenting with severe febrile illness in a rural area with seasonal P. falciparum transmission. Few studies addressed the use of malaria RDTs in children suspected of severe malaria and those who did recommend *Pf*HRP2-detecting RDTs which we expect to be less useful in high endemic settings.

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Chapter 2. *P. falciparum* samples generating false positive *P. vivax* lines

Malaria rapid diagnostic tests: *Plasmodium falciparum* infections with high parasite densities may generate false positive *Plasmodium vivax* pLDH lines

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Malaria rapid diagnostic tests: *Plasmodium falciparum* infections with high parasite densities may generate false positive *Plasmodium vivax* pLDH lines

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Abstract

Background: Most malaria rapid diagnostic tests (RDTs) detect *Plasmodium falciparum* and an antigen common to the four species. *Plasmodium vivax*-specific RDTs target *P. vivax*-specific parasite lactate dehydrogenase (Pv-pLDH). Previous observations of false positive Pv-pLDH test lines in *P. falciparum* samples incited to the present study, which assessed *P. vivax*-specific RDTs for the occurrence of false positive Pv-pLDH lines in *P. falciparum* samples.

Methods: Nine *P. vivax*-specific RDTs were tested with 85 *P. falciparum* samples of high (≥2%) parasite density. Mixed *P. falciparum/P. vivax* infections were ruled out by real-time PCR. The RDTs included two-band (detecting Pv-pLDH), three-band (detecting *P. falciparum*-antigen and Pv-pLDH) and four-band RDTs (detecting *P. falciparum*, Pv-pLDH and pan-pLDH).

Results: False positive Pv-pLDH lines were observed in 6/9 RDTs (including two- three- and four-band RDTs). They occurred in the individual RDT brands at frequencies ranging from 8.2% to 29.1%. For 19/85 samples, at least two RDT brands generated a false positive Pv-pLDH line. Sixteen of 85 (18.8%) false positive lines were of medium or strong line intensity. There was no significant relation between false positive results and parasite density or geographic origin of the samples.

Conclusion: False positive Pv-pLDH lines in *P. falciparum* samples with high parasite density occurred in 6/9 *P. vivax*-specific RDTs. This is of concern as *P. falciparum* and *P. vivax* are co-circulating in many regions. The diagnosis of life-threatening *P. falciparum* malaria may be missed (two-band Pv-pLDH RDT), or the patient may be treated incorrectly with primaquine (three- or four-band RDTs).

Background

Malaria rapid diagnostic tests (RDTs) are immunochromatographic tests targeting antigens of one or more *Plasmodium* species. Signals are visible as cherry-red to purple coloured lines, comprising a control line (which indicates that the test has been performed well) and one or two test lines. The initially developed two band tests generate a test line that targets *P. falciparum* by detecting either histidine-rich protein 2 (HRP-2) or *P. falciparum*-specific parasite lactate dehydrogenase (Pf-pLDH). The later developed three band tests include a second target that is common to the four *Plasmodium* species, such as aldolase or pan-specific parasite lactate dehydrogenase (pan-pLDH). However, the conventional three-band RDTs, detecting a *P. falciparum*-specific antigen and a pan-*Plasmodium* antigen, cannot distinguish between a *P. falciparum* infection and a mixed infection with *P. vivax* when both test lines are observed. Differentiation between the non-*falciparum* species is neither possible. *P. falciparum* and *P. vivax* infections require different treatment, which makes discrimination between the two species important. RDTs specific to *P. vivax* could be useful. There are two-band RDTs that detect *Plasmodium vivax*-specific pLDH (Pv-pLDH), three-band RDTs in which Pv-pLDH is combined with HRP-2 or Pf-pLDH, and so-

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called four-band tests that combine detection of HRP-2, pan-pLDH and Pv-pLDH. The Pv-pLDH tests have undergone limited evaluation [1-3].

In previous evaluations of RDTs targeting Pv-pLDH, rare but consistent false positive Pv-pLDH test lines were observed among *P. falciparum* samples, especially at high parasite densities [2-4]. These observations are of concern as this limits the potential use of the Pv-pLDH directed RDTs, both in endemic and non-endemic settings. In order to study the extent of this phenomenon among other RDTs, the present study was undertaken to challenge available RDT brands with a Pv-pLDH test line to a panel of *P. falciparum* samples with high parasite densities.

Methods

Study design

Several RDTs were retrospectively evaluated in a reference laboratory on a panel of stored whole blood samples obtained in patients suspected of malaria. The reference method was microscopy corrected by polymerase chain reaction (PCR).

Patient samples

In this study stored whole blood samples (-70°C) were used, obtained in returned international travelers suspected of malaria presenting at the outpatient department of the Institute of Tropical Medicine (ITM), Antwerp, Belgium, or submitted by other Belgian laboratories to ITM in its function of National Reference Center. Samples had been obtained between 1996 and 2009 and were classified in regions of travel destinations according to the United Nations classification of geographical region and composition [5]. All samples were evaluated by microscopy and real-time PCR for species identification (ruling out mixed infections) and determination of parasite density, as described previously [6,7]. For the purpose of this study, the more convenient parasite density expressed by % of infected red blood cells was applied, thereby assuming 50,000/μl to be equal to 1% of red blood cells parasitized [8]. *P. falciparum*-infected samples with parasite densities ≥2% (≥100,000/μl) were selected.

Malaria rapid diagnostic tests

RDTs containing a *P. vivax*-specific test line were selected, including those published on the World Health Organization (WHO) list of RDT manufacturers with adequate evidence of good manufacturing [9] as well as others available on the international market. We checked the package inserts to ensure the antigen used was *P. vivax*-specific. In line with other comparative evaluations [7,10] it was decided not to display individual RDT brand names because of the wide lot-to-lot variability and the frequent changes in composition and brand names and types [1].

Test procedures

Tests were performed according to the manufacturers' instructions, except that a pipette (Finnpipette, Helsinki, Finland) was used instead of the transfer devices supplied by the manufacturer. The laboratory technicians involved in the study have received a detailed training and their performance and agreement are monitored by participation to internal and external quality control assessments. Readings were done by three subsequent observers, of whom the first always was the one performing the test, and carried out at daylight assisted by an electric bulb. The observers were blinded to each others readings and to the results of microscopy and PCR. In case no control line was observed the test was considered invalid and repeated. Test line intensities were scored according to a system of five categories as described previously [6]: none (no line visible), faint (barely visible line), weak (paler than the control line), medium (equal to the control line) and strong (stronger than the control line). Test results were based on consensus agreement: the same test result observed by at least two out of three readers. In case of no consensus the result of the first reader was considered. Pv-pLDH lines generated by *P. falciparum* samples will be further referred to as false positive Pv-pLDH lines.

Statistical analysis

The nonparametric Spearman's rank correlation coefficient r_s was used to measure the strength of association between parasite density and the number of RDTs with a false positive Pv-pLDH test line. Associations were considered significant at a p-value < 0.05. Inter-observer agreement for line intensities and positive and negative test results were expressed by kappa values for each pair of observers and by the percentage of overall agreement between the three observers.

Ethical review

The study was reviewed and approved by the Institutional Review Board of ITM and by the Ethical Committee of Antwerp University, Belgium.

Results

Collection of samples and RDTs

Eighty-five *P. falciparum* samples with a parasite density ≥2% (100,000/μl) were selected. The parasite densities ranged from 2-35%, with 30 samples of at least 20% parasite density.

The male:female ratio was 3:1, with a median age of 40 years (range 3-98 years) and three children were under the age of five years. Samples were obtained in West Africa (n = 34), Middle Africa (n = 22), East Africa (n =

5), Southern Africa (n = 4) and West Asia (n = 1). Of 19 samples no data on the geographic origin were known and could not be retrieved. Four RDT brands (numbers. 1, 4, 7 and 9) were tested with less than 85 samples, due to either a lack of RDTs or a lack of sample.

Twenty different RDT brands were selected. Although their product name referred to *P. vivax*-specificity (for instance, by adding the epithet "Pf/Pv"), two of them in fact targeted pan-pLDH instead of Pv-pLDH: these RDTs were not considered for evaluation. Seven companies marketing eight RDT brands did not reply to the order of RDTs, despite several reminders via email contact. One *P. vivax*-specific three-band RDT was not included in this study because of bad clearance of the background, which made reading results impossible. The final panel consisted of nine different RDT brands from seven manufacturers, including one two-band (single Pv-pLDH test line), three three-band (Pv-pLDH and HRP-2 test line) and five four-band RDTs (Pv-pLDH, HRP-2 and pan-pLDH test line). Five RDTs had CE mark compliance and two were included in the WHO list of good manufacturing practices.

Three different four-band RDT brands (numbers 6, 7 and 8) had similar package inserts and the cassettes had identical morphology. Two three-band RDTs (numbers. 2 and 3) also had identical cassettes and similar package inserts. RDT numbers 1 and 4 were from the same manufacturer, as well as RDT numbers 2 and 5.

Test characteristics

There were no invalid test results. As expected, all the *P. falciparum* samples showed positive test lines for the HRP-2 and the pan-pLDH test lines, if present on the cassette. Table 1 lists the results for the Pv-pLDH test lines, expressed by line intensities. In total there were 85 false positive Pv-pLDH lines in six RDT brands, caused by a total of 42 samples. In the individual RDT brands they occurred at frequencies of 8.2% (7/85 samples) up to 29.1% (23/79 samples), among two-, three- and four-band tests. There was no difference between RDTs that were CE-marked or WHO-listed and those which were not.

Table 2 lists the details of consensus readings of line intensities for the false positive Pv-pLDH test lines, according to parasite densities and region of infection. In 2/736 readings there was no consensus and the results of the first reader were considered. Nineteen samples generated a false positive line in at least two RDTs. There was no apparent relation between parasite density of samples and the occurrence of false positive Pv-pLDH lines (r_s = 0,155, p = 0,153). False-positive Pv-pLDH lines occurred exclusively in samples from patients returning from Middle or West Africa (Table 3), but there was no significant relation between geographic origin of samples and false-positive results.

Most (69/85, 81.2%) false-positive Pv-pLDH readings were faint or weak (Table 1), but strong line intensities were observed in two RDT brands (Tables 1 and 2). Taken all readings together, inter-observer agreements for line intensity readings were good for HRP-2, pan-pLDH and Pv-pLDH test lines. For the Pv-pLDH line, in terms of positive and negative readings for all brands together, kappa values between pairs of observers were good (0.79, 0.70, 0.77) and overall agreement was excellent (88.0%). For the RDT brands considered separately, overall agreement for the Pv-pLDH line ranged from 75.9% to 100%.

Discussion

In this study, six out of nine *P. vivax*-specific RDTs showed false positive Pv-pLDH lines when challenged to a panel of 85 *P. falciparum* samples with high (≥2%) parasite densities, in which mixed infections with *P. vivax*

Table 1: Line intensity reading for Pv-pLDH lines in *P. falciparum* samples with parasite densities ≥2%

			Line intensity readings, number of samples				
Nr	**Type**	**Nr of samples tested**	**Faint**	**Weak**	**Medium**	**Strong**	**Total positive (% of total samples)**
1	Two-band	84	7	4	1	4	16 (19.0%)
2	Three-band	85					0
3	Three-band	85					0
4	Three-band	66	2		5	4	11 (16.7%)
5	Four-band	85					0
6	Four-band	85	8	13			21 (24.7%)
7	Four-band	79	19	3	1		23 (29.1%)
8	Four-band	85	2	5			7 (8.2%)
9	Four-band	82	1	5	1		7 (8.5%)

Two-band: Pv-pLDH test line; Three-band: Pv-pLDH and HRP-2 test line; Four-band: Pv-pLDH, HRP-2 and pan-pLDH test line.

Table 2: False positive Pv-pLDH lines in 42 *P. falciparum* samples with parasite densities ≥2%

Samples			RDT number and type						
Nr	**% parasite density**	**Origin**	**1. Two-band**	**4. Three-band**	**6. Four-band**	**7. Four-band**	**8. Four-band**	**9. Four-band**	**Total RDTs positive**
1	2,0	MAF	-	-	-	-	-	W	1
2	2,2	MAF	-	*	F	-	-	-	1
3	2,3	MAF	-	*	W	-	-	-	1
4	2,8	WAF	F	F	-	-	-	-	2
5	3,2	MAF	W	S	W	W	W	W	6
6	3,2	MAF	-	*	-	F	F	-	2
7	3,3	WAF	-	*	F	-	-	-	1
8	3,7	ND	S	*	-	F	-	-	2
9	3,8	ND	-	-	-	F	-	-	1
10	3,9	ND	-	-	W	F	-	-	2
11	4,0	WAF	S	S	W	F	W	-	5
12	4,2	WAF	W	M	-	-	-	-	2
13	4,5	WAF	F	F	-	-	-	-	2
14	4,6	MAF	-	-	F	-	-	-	1
15	5,3	WAF	M	M	W	F	-	-	4
16	5,5	WAF	-	-	-	F	-	-	1
17	5,7	MAF	S	S	W	W	-	F	5
18	6,6	MAF	F	*	-	-	-	-	1
19	7,0	ND	-	-	W	F	-	-	2
20	7,2	WAF	S	*	W	M	W	M	5
21	7,4	WAF	-	-	-	F	-	-	1
22	7,6	MAF	-	-	-	F	-	-	1
23	9,1	ND	-	-	-	-	-	W	1
24	9,6	ND	*	M	W	F	-	-	3
25	20,0	ND	-	-	-	F	-	-	1
26	20,0	WAF	F	-	-	F	-	-	2
27	20,0	WAF	-	-	W	-	-	-	1
28	20,0	WAF	W	S	W	F	W	-	5
29	20,0	WAF	W	M	F	F	-	W	5
30	20,0	WAF	-	-	F	F	-	-	2
31	20,0	ND	F	-	-	-	-	-	1
32	20,0	ND	F	-	-	F	-	-	2
33	20,0	WAF	-	-	-	F	-	-	1
34	20,0	MAF	-	*	-	-	-	W	1
35	20,0	ND	-	*	F	-	-	-	1
36	20,0	MAF	F	*	-	-	-	-	1
37	20,0	WAF	-	*	W	-	-	-	1
38	20,0	WAF	-	*	F	-	-	-	1
39	20,0	WAF	-	*	F	-	-	-	1
40	20,0	MAF	-	-	-	F	-	-	1
41	20,0	WAF	-	M	W	W	W	-	4

Table 2: False positive Pv-pLDH lines in 42 *P. falciparum* samples with parasite densities ≥2% (Continued)

42	35,0	WAF	-	*	-	-	F	-	1
Total false positive Pv-pLDH lines			16	11	21	23	7	7	85

MAF = Middle Africa; WAF = West Africa; ND = No Data
F = faint; W = weak; M = medium; S = strong; - = negative; * = sample not tested due to lack of RDT or sample

were excluded by PCR analysis. Frequencies for individual brands ranged from 8.2% (7/85 samples) to 29.1% (23/79 samples).

Plasmodium vivax accounts for almost half of the malaria infections worldwide and is no longer considered as a mild infection: complicated infections have been demonstrated in both endemic countries and in returned travelers [11,12]. In addition, *P. vivax* malaria may be chloroquine resistant and has a tendency to relapse [13]. To eradicate the dormant liver stages, primaquine treatment is needed. Primaquine is contraindicated in case of glucose-6-phosphate dehydrogenase (G6PD) deficiency, due to the risk of hemolysis [14,15]. G6PD deficiency is common in most *P. vivax* malaria areas; moreover, in these areas G6PD testing is impractical due to a lack of funds, equipments or expertise [16].

RDTs detecting *P. vivax*-specific pLDH are of additional value for the diagnosis of malaria in both *P. vivax* endemic areas and in the setting of travel medicine. The conventional three-band RDTs detecting a *P. falciparum*-specific antigen and a pan-*Plasmodium* antigen cannot distinguish between a *P. falciparum* infection and a mixed infection with *P. vivax* when both test lines are observed. Differentiation between the non-*falciparum* species is neither possible. Three- or four-band RDTs that target Pv-pLDH have the advantage that they can detect *P. vivax* in mixed infections: they are an adjunct to microscopy as *P. vivax* is often microscopically under diagnosed in mixed infections [13]. In addition they can be used to distinguish between *Plasmodium ovale* and *P. vivax* [2]. This is an advantage in the non-endemic setting, where microscopic differentiation between *P. ovale* and *P. vivax* is notoriously difficult [17].

Plasmodium vivax-specific RDTs have hardly been evaluated [18-22], and are not discussed in any of the recent reviews on malaria RDTs [1,8,17,23]. The present findings are of concern particularly in areas where *P. falciparum* and *P. vivax* are co-circulating: in case of a two-band Pv-pLDH test, the diagnosis of life-threatening *P. falciparum* malaria may be missed, which is especially of concern in remote areas among less experienced staff and without backup of microscopy. In case of a three- or four-band RDT, the patient may be treated incorrectly with primaquine, leading to severe hemolysis in patients with G6PD-deficiency.

Among the limitations of the present study, we should mention its retrospective nature (precluding further work-up of samples), the test conditions in a reference laboratory (which are more favorable than those in field settings) and the fact that all but one *P. falciparum* samples were obtained in travelers returning from Africa. In addition, the present study explored false positive Pv-

Table 3: Numbers of *P. falciparum* samples generating false positive Pv-pLDH lines according to geographic origin

Origin (region)	Numbers of samples tested*		
	Total	Negative	Positive** (% of total samples)
East Africa	5	5	0
Middle Africa	22	10	12 (54.5)
Southern Africa	4	4	0
West Africa	34	14	20 (58.8)
West Asia	1	1	0
No data	19	9	10 (52.6)
All samples	85	43	42 (49.4)

* Nine RDT brands were tested with a panel of 85 samples
** Defined as a sample with a false positive Pv-pLDH line in ≥1 RDT brand

pLDH lines exclusively in samples with high *P. falciparum* parasite densities, in line with the previous observations [2-4]. However, the inclusion of *P. falciparum* samples of lower parasite density and *Plasmodium* negative samples would have completed the picture. A prospective study in an area with *P. falciparum* and *P. vivax* coexistence should be performed to assess the relevance of the false positive Pv-pLDH lines in a field setting.

False-positive reactions of *P. vivax* samples with the HRP-2 line and the Pf-pLDH line have been described previously [24-26], but the presently observed false-positive Pv-pLDH lines in *P. falciparum* samples have only been reported anecdotally [2-4,22]. Among the panel of RDTs tested in the WHO and Foundation for Innovative New Diagnostics (FIND) study, there were two RDTs with a Pv-pLDH line: according to the tables, one of them generated false positive Pv-pLDH lines in 1.9% (6/316) of the *P. falciparum* samples tested.

Without exact knowledge of the antibodies targeting the Pv-pLDH antigen, it is difficult to explain the phenomenon, especially since false positive Pv-pLDH lines in the present study occurred not consistently among the different samples and brands. Weak cross-reactions may also be facilitated by slight differences in the composition of the nitrocellulose membrane and the diluent. It is known that several companies use the same pLDH antibodies in their RDT brands [27] and this may also be the case for Pv-pLDH: this is reflected by the identical product presentations and similar false positive rates of RDT brands numbers 6 and 7 and numbers 2 and 3 respectively. In the present study, there was no apparent relation between the false positive Pv-pLDH lines and the geographic origin of the infection, but it should be noted that mainly samples from sub-Saharan Africa were included.

The present study also revealed problems in RDT availability and communication with the supplier in the international market, as eight brands were not delivered despite several reminders. Furthermore, two of the RDTs that claimed to be *P. vivax*-specific by their label and name proved to detect pan-pLDH according to their package insert. Similar confusing names have been noted previously, also for the antibodies targeting aldolase [28].

Conclusion

The occurrence of false positive Pv-pLDH lines in *P. falciparum* samples with high parasite densities was observed in six out of nine *P. vivax*-specific RDTs, in two-, three- and four-band RDTs. The false positive Pv-pLDH lines are of concern because the diagnosis of life-threatening *P. falciparum* malaria may be missed (two-band Pv-pLDH RDT), or the patient will be treated incorrectly with primaquine (three- or four-band RDT), which may cause severe hemolysis in patients with G6PD-deficiency. A prospective study in an area with *P. falciparum* and *P. vivax* coexistence should be performed to assess the relevance of the false positive Pv-pLDH lines in a field setting.

List of abbreviations

FIND: Foundation of New Innovative Diagnostics; G6PD: Glucose-6-phosphate dehydrogenase; HRP-2: Histidine-rich protein 2; ITM: Institute of Tropical Medicine; *P.*: *Plasmodium*; pan-pLDH: pan *Plasmodium*-specific parasite lactate dehydrogenase; PCR: Polymerase chain reaction; Pf-pLDH: *Plasmodium falciparum*-specific parasite lactate dehydrogenase; pLDH: *Plasmodium*-specific parasite lactate dehydrogenase; Pv-pLDH: *Plasmodium vivax*-specific parasite lactate dehydrogenase; RDT(s): Rapid diagnostic test(s); WHO: World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PG and JJ designed the study protocol. MvE and JvdE organized prospective sample collection. JM and PG carried out the test evaluations, LC performed PCR analysis. JM, PG and JJ analyzed and interpreted the results and JM and JJ drafted the manuscript. JM and PG performed statistical analysis. All authors read and approved the final manuscript.

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Chapter 3. Which RDT for the Peruvian Amazon?

Rapid diagnostic tests for malaria diagnosis in the Peruvian Amazon: impact of pfhrp2 gene deletions and cross-reactions

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Rapid Diagnostic Tests for Malaria Diagnosis in the Peruvian Amazon: Impact of *pfhrp2* Gene Deletions and Cross-Reactions

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Abstract

Background: In the Peruvian Amazon, *Plasmodium falciparum* and *Plasmodium vivax* malaria are endemic in rural areas, where microscopy is not available. Malaria rapid diagnostic tests (RDTs) provide quick and accurate diagnosis. However, *pfhrp2* gene deletions may limit the use of histidine-rich protein-2 (PfHRP2) detecting RDTs. Further, cross-reactions of *P. falciparum* with *P. vivax*-specific test lines and vice versa may impair diagnostic specificity.

Methods: Thirteen RDT products were evaluated on 179 prospectively collected malaria positive samples. Species diagnosis was performed by microscopy and confirmed by PCR. *Pfhrp2* gene deletions were assessed by PCR.

Results: Sensitivity for *P. falciparum* diagnosis was lower for PfHRP2 compared to *P. falciparum*-specific *Plasmodium* lactate dehydrogenase (Pf-pLDH)- detecting RDTs (71.6% vs. 98.7%, $p<0.001$). Most (19/21) false negative PfHRP2 results were associated with *pfhrp2* gene deletions (25.7% of 74 *P. falciparum* samples). Diagnostic sensitivity for *P. vivax* (101 samples) was excellent, except for two products. In 10/12 *P. vivax*-detecting RDT products, cross-reactions with the PfHRP2 or Pf-pLDH line occurred at a median frequency of 2.5% (range 0%–10.9%) of *P. vivax* samples assessed. In two RDT products, two and one *P. falciparum* samples respectively cross-reacted with the Pv-pLDH line. Two Pf-pLDH/pan-pLDH-detecting RDTs showed excellent sensitivity with few (1.0%) cross-reactions but showed faint Pf-pLDH lines in 24.7% and 38.9% of *P. falciparum* samples.

Conclusion: PfHRP2-detecting RDTs are not suitable in the Peruvian Amazon due to *pfhrp2* gene deletions. Two Pf-pLDH-detecting RDTs performed excellently and are promising RDTs for this region although faint test lines are of concern.

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Introduction

In Peru, malaria is mainly endemic in the Amazon region, where it is the primary cause of morbidity in adults and the fourth in children [1]. According to the recommendations of the World Health Organization (WHO), diagnosis and treatment should be based on parasitological confirmation by either microscopy or malaria rapid diagnostic tests (RDTs) [2]. In Peru, most cases occur in rural areas where no microscopy is available. Currently, thick blood films (TBFs) of malaria suspected patients are sent for analysis to the most nearby health center, but this process takes several days and patients are often treated presumptively [3]. In such conditions RDTs could be useful, providing quick and accurate diagnosis, thereby leading to timely and correct treatment and reducing the severity and economic burden of disease. Besides, use of RDTs in the Peruvian Amazon has been demonstrated to be cost-effective [4].

RDTs are handheld cassettes detecting malaria parasites by an antigen-antibody reaction on a nitrocellulose strip which become visible as blue or cherry-red test lines. There are several detection antibodies, directed to different antigens: histidine-rich protein-2 (PfHRP2) and *Plasmodium falciparum*-specific *Plasmodium* lactate dehydrogenase (Pf-pLDH) for *P. falciparum*; *Plasmodium vivax*-specific pLDH (Pv-pLDH) for *P. vivax*, and pan-pLDH and aldolase which are common to all four *Plasmodium* species.

The occurrence of both *P. vivax* and *P. falciparum* in Peru requires an RDT type that detects and differentiates between both species as they require different treatment [2]. However, cross-reactions may occur, *i.e.* the presence of a visible *P. falciparum* test line among *P. vivax* samples and vice versa [5,6], due to genuine antigen-antibody interactions or non-specific bindings [7]. In addition, *P. falciparum* parasites lacking the *pfhrp2* and *pfhrp3* genes, -encoding PfHRP2 and the related protein PfHRP3 respectively-

have been recently described in Peru [8] indicating that the use of PfHRP2 detecting RDTs may be limited [8]. Previous evaluations of two PfHRP2 detecting RDTs in Peru demonstrated sensitivity for *P. falciparum* diagnosis of 95% [9] and 53.5% [10].

The aims of the present study were to assess diagnostic accuracy of a panel of different RDT products for malaria diagnosis in the Peruvian Amazon, with particular focus on the impact of *pfhrp2* and *pfhrp3* gene deletions on diagnostic sensitivity and of cross-reactions on diagnostic specificity.

Methods

Ethics statement

The study was approved by the Ethical Review Board of the Universidad Peruana Cayetano Heredia, Lima, Peru (Code SIDISI: 55587 and 55239). All patients with a positive TBF, performed as part of routine patient care, were included after signing informed consent. Written informed consent was obtained from the patient himself in the case of adults or from the parent/guardian in case of a minor (<18 years).

Study site and population

Several health centers around Iquitos (Figure 1) were included. Malaria in the Peruvian Amazon is perennial with a peak during the rainy season (November – May) and an incidence of 10–50 malaria cases per 1000 inhabitants per year [11]. Patients were included by either passive case detection (symptomatic patients presenting at the health centers) or active case detection (outreach teams performing malaria screening in epidemic communities). All patients with a positive TBF were included after signing informed consent. Previous antimalarial treatment, symptoms and travel history were recorded.

Samples

EDTA anti-coagulated venous blood samples were drawn and transported to the laboratory of San Juan where RDTs were performed. After RDT performance, samples were aliquoted and stored at −20°C, usually within 24 hours (range 2–72 hours) after sample collection, pending further analysis.

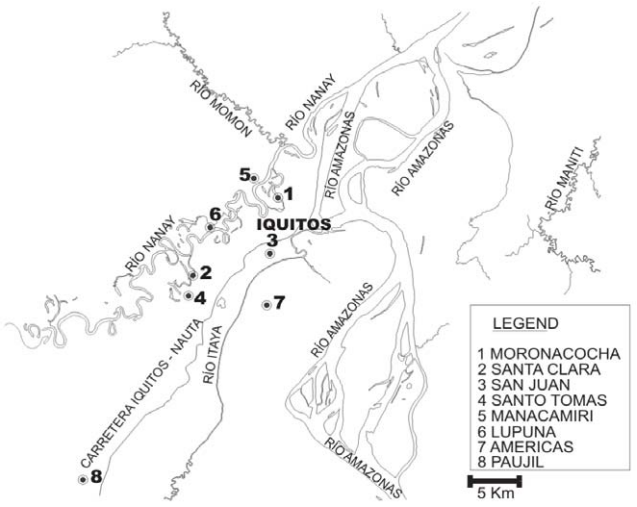

Figure 1. Map of included health centers. The village of Atalaya (−3.58, −73.75), located 59 km to the West of Iquitos, is not displayed on the map.
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Malaria rapid diagnostic tests

Thirteen RDT products detecting several target antigens were selected (Table 1), based on good performance as documented by the WHO/Foundation for Innovative New Diagnostics (FIND) malaria RDT evaluation program [12,13] or recent release on the market.

Both SDFK90 and Paracheck detect only *P. falciparum* and were included for evaluation of *P. falciparum* diagnosis. SDFK90 was only performed on *P. falciparum* samples and mixed infections. RDTs were purchased at the Institute of Tropical Medicine (ITM), Belgium and shipped to Peru. For logistic reasons (delays of delivery and shipment), some RDTs had to be performed on stored samples, in these cases median period of sample storage was 51 days (range 29–131 days).

Test procedures

RDTs were performed according to the manufacturer's instructions except that the supplied transfer device was replaced by a micropipette. The first observer read test results within the specified reading time, the second and, when available, third observer within 10 additional minutes. Observers were blinded to each other's readings. In case of absence of the control line the test was repeated. A scoring system of five categories was used to assess line intensities [14]. Test results were based on consensus agreement in case of three observers. In all other cases, the result of the first observer was considered.

Microscopy

At the laboratory of San Juan, species and parasite density were determined by TBF microscopy, assuming a white blood cell count of 8,000/µl [15]. For quality control (QC), 20% randomly selected slides, including those with interpretive problems, discordant RDT results, negative slides and suspected mixed infections were reexamined by two blinded expert microscopists at ITM. For parasite density the results of the first microscopist were considered except when QC indicated a density of more than two fold difference with the original count, in such cases mean of the two QC readings was considered.

DNA extraction

DNA was extracted from 200 µl whole blood using QIAamp DNA blood Mini kit (QIAGEN, Venlo, The Netherlands), according to the manufacturer's instructions except for a dilution in 100 µl instead of 200 µl elution buffer.

Species-specific PCR

In case of discordances between RDT and microscopy or between initial and QC microscopy, real-time PCR (*P. falciparum*/*P. vivax*) was performed [16] which was considered conclusive.

Assessment of *pfhrp2* and *pfhrp3* gene deletions

Confirmed *P. falciparum* samples were assessed for *pfhrp2* and *pfhrp3* gene deletions by conventional PCR using primers and conditions as described elsewhere [8,17]. For *pfhrp2*, two amplifications were performed: one of entire exon 2 (encoding PfHRP2) and another across exon 1 and exon 2 (exon1–2). Samples were considered lacking the *pfhrp2* gene when both amplifications failed to generate a PCR product. For *pfhrp3*, a single amplification of entire exon 2 was performed.

PfHRP2 ELISA

The presence of PfHRP2 protein in whole blood samples was determined by enzyme linked immune sorbent assay (ELISA,

Table 1. Overview of RDT products and their lot numbers.

Product name	Manufacturer/distributor	Further referred to as	Target antigen Pf	Target antigen pan/Pv	Lot numbers	Recommended storage temperature
ADVANTAGE Mal Card	J. Mitra & Co., New Dehli, India	Advantage	Pf-pLDH	pan-pLDH	ACM171110	4–30°C
AZOG Malaria Pf/Pv	AZOG, Inc. New Jersey, USA	AZOG	PfHRP2	Pv-pLDH	58LAB017	2–30°C
CareStart™ Malaria Pf-pLDH/pLDH (Pf/PAN) Combo	Access Bio, Inc. New Jersey, USA	CareStart pLDH	Pf-pLDH	pan-pLDH	A10IL	4–30°C
CareStart™ Malaria HRP2/Pv-pLDH (Pf/Pv) Combo	Access Bio, Inc. New Jersey, USA	CareStart Pf/Pv	PfHRP2	Pv-pLDH	J10IV	4–30°C
Falcivax Rapid Test for Malaria Pv/Pf	Zephyr Biomedicals, Verna, India	Falcivax	PfHRP2	Pv-pLDH	81098	4–30°C
First Response Ag malaria pLDH/HRP2 combo test	Premier Medical Corporation Daman, India	First Response	PfHRP2	pan-pLDH	69I0610	4–30°C
Onsite Pf/Pv Ag rapid test	CTK Biotech, Inc. San Diego, USA	Onsite	PfHRP2	Pv-pLDH	F0810G2	2–30°C
PARACHECK Pf® (device)	Orchid Biomedical Systems Verna, India	Paracheck	PfHRP2	-	31795, 31797	4–45°C
Parascreen Rapid Test for Malaria Pan/Pf	Zephyr Biomedicals, Verna, India	Parascreen	PfHRP2	pan-pLDH	101176	4–30°C
SD Bioline Malaria Antigen test	Standard diagnostic, Hagal-dong, Korea	SDFK40	Pf-pLDH	pan-pLDH	MLRDT1001, MLRDT1002	1–40°C
SD Bioline Malaria Antigen P.f/pan	Standard diagnostic, Hagal-dong, Korea	SDFK60	PfHRP2	pan-pLDH	90026, 90017, 90096	1–40°C
SD Bioline Malaria Antigen P.f/P.v	Standard diagnostic, Hagal-dong, Korea	SDFK80	PfHRP2	Pv-pLDH	145015, 145016	1–40°C
SD Bioline Malaria Antigen P.f	Standard diagnostic, Hagal-dong, Korea	SDFK90	PfHRP2 and Pf-pLDH*	-	MFRDT1001, MFRDT1002	1–40°C

*SDFK90 contains 2 test lines specific for *P. falciparum*.

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Standard Diagnostic, Hagal-Dong, Korea) according to the manufacturer's instructions. ELISA was performed in all samples with *P. falciparum* infection, mixed infections and in *P. vivax* samples generating visible PfHRP2 lines.

Statistical analysis

Diagnostic sensitivity (calculated with 95% confidence intervals (C.I.)) of the RDT products was defined as the number of *P. falciparum* or *P. vivax* samples with a visible *P. falciparum*-specific or Pv-/pan-pLDH test line respectively (regardless of the presence of another test line), divided by the total number of *P. falciparum* or *P. vivax* samples respectively. Mixed infections were not included for calculation. Cross-reactions were defined as *P. falciparum* samples generating a visible Pv-pLDH line or *P. vivax* samples generating a visible PfHRP2 or Pf-pLDH line.

Proportions were assessed for statistical significance using the Chi-square test or, in case of small sample size, the Fisher-exact test. A p-value<0.05 was considered significant.

Interobserver agreement was determined by kappa values (κ) for positive and negative readings and line intensity readings between the first pair of observers.

Additional analysis

All microscopically confirmed *P. falciparum* samples that did not show a visible PfHRP2 line in more than one RDT product were repeated two times with all PfHRP2-detecting RDTs.

Results

Patients and samples

From December 2010–July 2011, 182 patients were included, in three patients malaria was not confirmed by microscopy nor by PCR. Final sample collection consisted of *P. falciparum* (n = 74), *P. vivax* (n = 101) and four mixed infections. The collected samples comprised 5% of all *P. falciparum* and *P. vivax* infections reported in Loreto region in that time period [1,18]. Data of demography and parasite density are shown in Table 2. Nineteen patients, including the two asymptomatic cases, were included through active case detection performed once in Tarapoto (n = 5) and once in Atalaya (n = 14).

Diagnostic sensitivity of the RDT products

PfHRP2-detecting RDTs had significantly lower sensitivity for *P. falciparum* diagnosis compared to Pf-pLDH-detecting RDTs ($p<0.0001$, Table 3), due to a subset of samples that consequently failed to generate a PfHRP2 line in all PfHRP2 RDT products tested, see results below.

For *P. vivax* diagnosis, most RDTs performed equally well, except for AZOG (detecting Pv-pLDH) and Parascreen (detecting pan-pLDH) (Table 3), which failed to detect *P. vivax* samples at a median parasite density of 1,075/µl (range 255–4,532/µl) and 600.5/µl (range 255–10,720/µl) respectively.

The mixed infections were detected by all RDT products except for AZOG which displayed a single PfHRP2 line for a sample consisting predominantly of *P. falciparum* parasites.

Table 2. Patient data and parasite density of the final sample collection.

	P. falciparum (n = 74)	*P. vivax* (n = 101)	Mixed infection (n = 4)
Sample collection period	**Dec 2010–Jul 2011**	**Dec 2010–Mar 2011**	**Dec 2010–Mar 2011**
Male gender	41 (55.4%)	52 (51.5%)	4 (100%)
Age, median years (range)	27.5 (4–74)	29 (2–76)	31.5 (4–47)
Children <15 years, number (%)	16 (21.6%)	24 (23.8%)	1 (25%)
Median parasite density/µl (range)	4,971.5 (0–78,208)	5,080 (255–58,880)	9,527.5 (5,204–22,321)
Asymptomatic patients (number)	1 (1.4%)	1 (1.0%)	0 (0.0%)
Antimalarial treatment past 2 weeks	4 (5.4%)*	0 (0.0%)	0 (0.0%)

*artesunate + mefloquine since 2 days (n = 1), chloroquine since 2 days (n = 2), full course of chloroquine/primaquine (n = 1) at least >1 week ago (exact date not known).

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For *P. falciparum*, faint test line intensities occurred more frequently among Pf-pLDH compared to PfHRP2-detecting RDTs ($p<0.001$, Table 3). For *P. vivax*, no overall difference in proportion of faint test lines was observed between Pv-pLDH versus pan-pLDH-detecting RDTs.

Failure of *P. falciparum* diagnosis by PfHRP2-detecting RDTs and *pfhrp2* gene deletions

All PfHRP2-detecting RDTs failed to diagnose 21 *P. falciparum* samples (Table 4), whereas the Pf-pLDH-detecting RDTs detected all of them. Most samples (19/21) were lacking *pfhrp2* (no amplification of exon1–2 and exon2). The remaining two samples (PI151 and PI156) generated PCR products for *pfhrp2* exon1–2 and exon2. *Pfhrp2* gene deletions occurred at both low and high parasite densities (Table 4) and all patients were symptomatic. PfHRP2 ELISA of the 21 samples confirmed the absence of PfHRP2, with only one sample (PI26) showing a weak positive result (optical density ten-fold lower than other ELISA positive samples).

Pfhrp2: percentage of samples with gene deletions and geographic origin

Pfhrp2 gene deletions occurred among 19 (25.7%) *P. falciparum* samples. Thirteen (68.4%) were obtained from patients presenting at the health center of Santa Clara (Figure 2), with most patients

Table 3. Sensitivity, faint line intensity and cross-reactions of the different RDT products for detection of *P. falciparum* and *P. vivax*.

	% Sensitivity (95% C.I.)		% of positive test lines with faint intensity*		Number of cross-reactions (%)	
RDT product	***P. falciparum* (n = 74)**	***P. vivax* (n = 101)**	**PfHRP2 /Pf-pLDH**	**Pv-/pan-pLDH†**	***P. vivax* with PfHRP2/Pf-pLDH test line**	***P. falciparum* with Pv-pLDH test line**
PfHRP2-detecting RDT						
Paracheck	70.3 (58.5–80.3)	-	5.8	-	0 (0.0)	-
PfHRP2 and pan-pLDH detecting RDTs						
First Response	71.6 (60.0–81.5)	100.0 (94.6–100.0)	3.8	2.0	3 (3.0)	-
Parascreen	71.6 (60.0–81.5)	89.1 (81.4–94.4)	1.9	21.1	7 (6.9)	-
SDFK60	71.6 (60.0–81.5)	100.0 (94.6–100.0)	7.1	4.0	5 (5.0)	-
PfHRP2and Pv-pLDH detecting RDTs						
AZOG	71.6 (60.0–81.5)	87.1 (79.0–93.0)	17.0	79.5	2 (2.0)	0 (0.0)
CareStart Pf/Pv	71.6 (60.0–81.5)	100.0 (94.6–100.0)	5.7	10.9	11 (10.9)	0 (0.0)
Falcivax	71.6 (60.0–81.5)	100.0 (94.6–100.0)	1.9	7.9	5 (5.0)	0 (0.0)
Onsite	71.6 (60.0–81.5)	100.0 (94.6–100.0)	5.7	4.0	0 (0.0)	2 (2.7)
SDFK80	71.6 (60.0–81.5)	100.0 (94.6–100.0)	1.9	0.0	1 (1.0)	1 (1.4)
Pf-pLDH and pan-pLDH detecting RDTs						
Advantage	98.7 (92.7–100.0)	100.0 (94.6–100.0)	24.7	4.0	1 (1.0)	-
CareStart pLDH	98.7 (92.7–100.0)	99.0 (94.6–100.0)	9.6	8.0	10 (9.9)	-
SDFK40	97.3 (90.6–99.7)	100.0 (94.6–100.0)	38.9	1.0	1 (1.0)	-
PfHRP2 and Pf-pLDH detecting RDT						
SDFK90 PfHRP2 line	71.6 (60.0–81.5)	-	1.9	-	-	-
SDFK90 Pf-pLDH line	98.7 (92.7–100.0)	-	40.5	-	-	-

*cross-reactions excluded.

†only *P. vivax* samples were considered.

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Table 4. *P. falciparum* samples not detected by PfHRP2-detecting RDTs: *pfhrp2* and *pfhrp3* PCR results and PfHRP2 ELISA results.

Sample and patient information							
Sample number	**Sex**	**Age**	**Parasite density (/μl)**	***pfhrp2* exon 1–2**	***pfhrp2* exon 2**	***pfhrp3* exon 2**	**PfHRP2 ELISA**
PI138	f	56	0*	−	−	−	−
PI139	m	6	79	−	−	−	−
PI137	m	41	80	−	−	−	−
PI136	m	53	270	−	−	−	−
PI 24	f	20	752	−	−	−	−
PI113	f	30	876	−	−	−	−
PI142	m	21	1,000	−	−	−	−
PI151	m	28	1,222	+	+	−	−
PI 18	f	12	1,400	−	−	−	−
PI 78	m	37	2,808	−	−	−	−
PI156	f	36	3,480	+	+	+	−
PI135	m	7	4,784	−	−	−	−
PI153	m	70	5,080	−	−	−	−
PI140	f	20	5,640	−	−	−	−
PI 26	m	48	7,227	−	−	−	+/−
PI 27	f	67	7,840	−	−	−	−
PI163	m	65	16,552	−	−	−	−
PI 81	m	46	18,800	−	−	−	−
PI148	f	34	19,600	−	−	−	−
PI 74	m	38	22,560	−	−	−	−
PI 65	m	27	43,089	−	−	−	−

+ = positive, − = negative, +/− = weak positive.
*This sample contained only gametocytes.
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living in Tarapoto (8/13, 61.5%). The remaining six were distributed among three other health centers (Figure 2). *Pfhrp2* gene deletions were found throughout the study period and sometimes *P. falciparum* samples with and without *pfhrp2* gene deletions were found simultaneously in the same village. One patient with a *pfhrp2* gene deletion diagnosed at Morona Cocha had been travelling to Angamos (close to the Brazilian border) during the month previous to sampling.

Pfhrp3 gene deletion

In total 34 (43.6%) *P. falciparum* samples lacked the *pfhrp3* gene: they included all samples lacking the *pfhrp2* gene (n = 19) as well as 15 additional samples which contained *pfhrp2*, and which were correctly diagnosed by all PfHRP2-detecting RDTs.

Occurrence of cross reactions

In most (10/12) RDT products that were assessed with *P. vivax* samples, *P. falciparum* test lines (either PfHRP2 or Pf-pLDH) were visible at a median frequency of 2.5% (range 1.0%–10.9%). In total, 27 (26.7%) *P. vivax* samples were involved. In all of these samples, mixed infection with *P. falciparum* was excluded by PCR and none of the patients had reported *P. falciparum* infection in the month prior to sampling. In six of these samples however, HRP2 ELISA yielded a weak positive result. There was no apparent relation between parasite density and the occurrence of cross-reactions (range 255–58,880/μl).

In two RDT products, *P. falciparum* samples generated a visible Pv-pLDH line: one faint line for SDFK80 (parasite density 78,208/μl); and a faint and medium line for Onsite (parasite density 53,333/μl and 3,480/μl). Mixed infection with *P. vivax* was excluded by PCR and none of the patients reported recent *P. vivax* infection.

Interobserver agreement

For positive/negative readings, median κ per RDT product was 1.00 (range 0.84–1.00). For line intensity readings, median κ was 0.87 (range 0.62–0.99).

Discussion

The present study evaluated a panel of RDT products for malaria diagnosis in the Peruvian Amazon. It showed that Pf-pLDH-detecting RDTs performed significantly better for *P. falciparum* diagnosis compared to PfHRP2-detecting RDTs in this geographical region. The low sensitivity of PfHRP2-detecting RDTs was related to *pfhrp2* gene deletions which invariably leaded to false negative PfHRP2 results irrespective of the parasite density. For *P. vivax* diagnosis all but two RDT products performed well with no overall difference in sensitivity and line intensity between Pv-pLDH and pan-pLDH detecting RDTs. Cross-reactions with the *P. falciparum* line were observed in 10/12 *P. vivax*-detecting RDT products at a median frequency of 2.5% (range 1.0%–10.9%) of *P. vivax* samples assessed. In two RDT products, false positive Pv-pLDH lines were observed in up to 2.7% of *P. falciparum* samples.

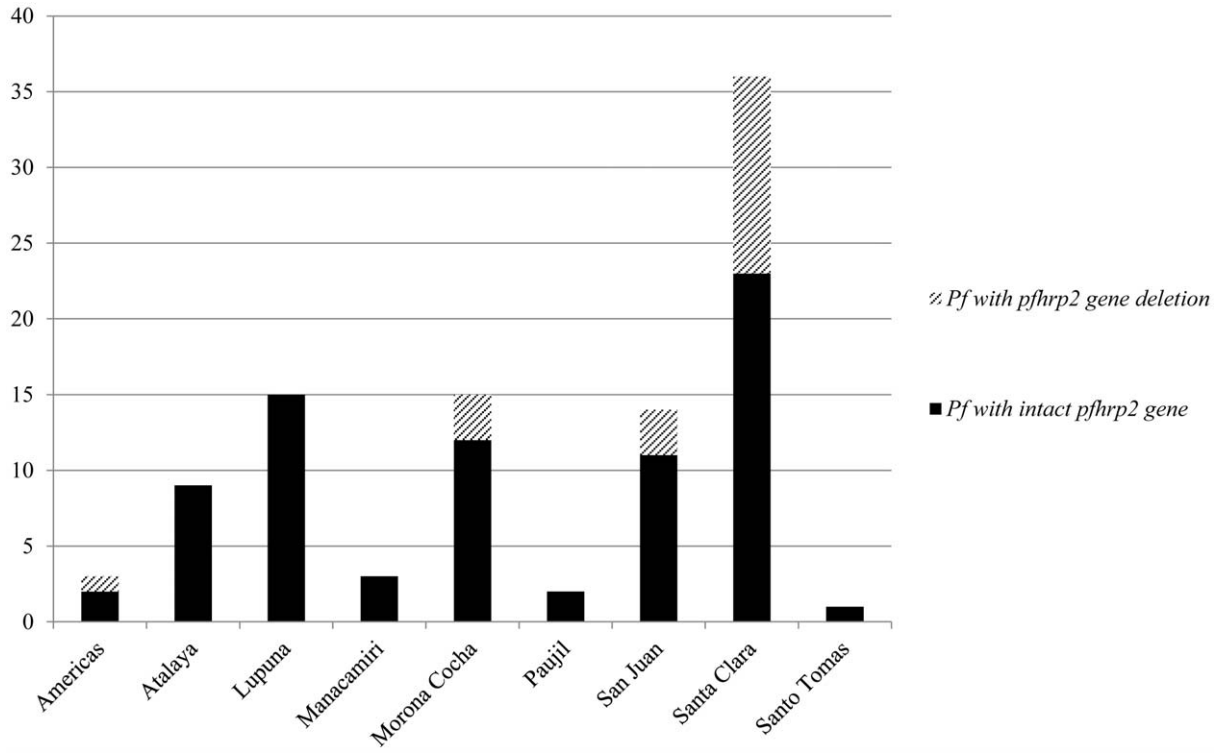

Figure 2. Number of *P. falciparum* samples containing or lacking the *pfhrp2* gene per health center. The village of Atalaya is not a health center, but is displayed separately as all samples in Atalaya were collected by an outreach team during an epidemic.
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Impact of *pfhrp2* gene deletions

The exact incidence of *pfhrp2* gene deletions in the Peruvian Amazon is not known. We presently found 25.7% of *P. falciparum* samples lacking *pfhrp2*, in a previous study this was 41.0% [8]. In the present study *pfhrp2* gene deletions were found at different sites, but not at all health centers. *Pfhrp2* gene deletions have however been reported throughout the Peruvian Amazon [8] as well as in Brazil [19] and one of the presently included patients might have acquired infection near the Brazilian border. By consequence, the findings as currently described may be applicable to the whole Amazon region.

The impact of *pfhrp2* gene deletions is further highlighted by the fact that all samples lacking *pfhrp2* were not detected by any of the PfHRP2-detecting RDT products. In addition, all samples lacking *pfhrp2* were found in symptomatic patients and occurred at both high and low parasite densities, in contrast to a previous study [20] which demonstrated *pfhrp2* gene deletions only in asymptomatic patients and at low parasite densities. Of note is that in 1998–1999 an evaluation of the PfHRP2-detecting RDT Parasight-F around Iquitos showed sensitivity for *P. falciparum* diagnosis of 95% [9]. Possibly, *pfhrp2* gene deletions have become common in this area only recently.

Discordances between *pfhrp2* PCR and PfHRP2 RDT results

For samples PI151 and PI156, the presence of *pfhrp2* exon2 was demonstrated by both PCRs but PfHRP2 RDT and ELISA results were negative. Parasite density of both samples was far above the RDT detection threshold and does not explain failure of detection. A mutation or deletion may have occurred, leading to failure of production of the antigen or production of an antigen that is not recognized. Failure of detection of both samples may also be due to errors in transcription or translation, causing low parasite protein expression and consequently failure of detection by RDTs and ELISA [21]. Further research is needed to investigate the occurrence and cause of this phenomenon.

Role of *pfhrp3*

It has been postulated that PfHRP3 might compensate for absence of PfHRP2 in PfHRP2-detecting diagnosis, due to cross-reaction of PfHRP3 with PfHRP2 antibodies [8,17]. In the present study this could not be assessed since all *pfhrp2* negative samples lacked the *pfhrp3* gene as well.

Cross-reactions

In all samples showing cross-reactions, mixed infections were excluded and *Plasmodium* infection during the month previous to sampling was not reported. In the case of *P. vivax* samples generating a PfHRP2 line, past subclinical infection with PfHRP2 persistence (caused by slow clearance of PfHRP2 [22]) may have occurred in at least part of the samples, as supported by the weak positive ELISA results in six samples. However, optical density values in these samples were low and PfHRP2 lines were only visible in few RDT products, which makes non-specific reactions a more plausible explanation. In the case of visible Pf-pLDH lines among *P. vivax* samples and Pv-pLDH lines among *P. falciparum* samples, genuine antigen-antibody reactions [23] or non-specific reactions [7] may have occurred. Cross-reactions (false positive *P. falciparum* test lines) among *P. vivax* samples are particularly relevant in RDTs detecting pan-pLDH: in these cases RDT results are interpreted as *P. falciparum* infection and the patient will not be treated with primaquine, which is needed to eradicate the liver stages. Conversely, false positive Pv-pLDH test lines among *P.*

falciparum samples indicate mixed *P. falciparum*/*P. vivax* infection, which will lead to unnecessary treatment with primaquine.

Limitations

The present study did not include *Plasmodium* negative patients, precluding calculation of specificity and positive and negative predictive values. However, it provides relevant data about RDT diagnostic sensitivity and its relation with *pfhrp2* gene deletions, based upon which suitable RDTs can be selected. Further, we included a large panel of simple one-step RDT products but did not include RDTs with a more complex procedure such as the previously evaluated OptiMAL [24]. Not all RDTs could be performed on fresh samples, though samples had been stored for a short period and had not been exposed to repeated freezing and thawing. Besides, no apparent differences were found between RDT results on stored versus fresh samples. Finally, observers of RDT results were not always blinded to microscopy results provided by the health center.

Which RDT for the Peruvian Amazon?

From the present study it is clear that PfHRP2-detecting RDTs are not suitable for the Peruvian Amazon, due to the high prevalence of *P. falciparum* samples lacking the *pfhrp2* gene which was invariably associated with false negative results. *Pfhrp2* gene deletions occurred at all parasite densities and all patients were symptomatic. The three Pf-pLDH-detecting RDTs - all combining pan-pLDH - performed excellently for *P. falciparum* and *P. vivax* diagnosis. Among one of them however, an unacceptably high proportion of *P. vivax* samples generated cross-reactions with the Pf-pLDH line, impeding its use. For the remaining two, the high number of faint test lines is of concern as especially in field settings faint lines tend to be overlooked or disregarded as negative [25,26,27]. Besides, general limitations of Pf-pLDH-detecting RDTs are a lower sensitivity at low parasite densities [7,12,14] and less heat stability, although the latter is currently less important than originally described [7,14] and SDFK40 reports heat stability up to 40°C (Table 1).

Despite the excellent diagnostic accuracy of SDFK40 and Advantage in the present study, prospective field evaluation on all malaria suspected patients is needed to determine positive and negative predictive values and end user performance. In the long term, the development of an RDT targeting both Pf-pLDH and Pv-pLDH could be considered. Such a combination could, besides diagnosing each of both species, also differentiate between *P. falciparum* and mixed *P. falciparum*/*P. vivax* infections, but is not yet commercially available.

Author Contributions

Conceived and designed the experiments: JM DG PG JJ. Performed the experiments: JM LS LC PG. Analyzed the data: JM DG JB LC PG JJ. Wrote the paper: JM JJ. Coordinated sample collection: JM DG.

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Chapter 4. Completeness and adequacy of instructions and labeling of RDTs

Malaria rapid diagnostic kits: quality of packaging, design and labeling of boxes and components and readability and accuracy of information inserts.

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Malaria rapid diagnostic kits: quality of packaging, design and labelling of boxes and components and readability and accuracy of information inserts

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Abstract

Background: The present study assessed malaria RDT kits for adequate and correct packaging, design and labelling of boxes and components. Information inserts were studied for readability and accuracy of information.

Methods: Criteria for packaging, design, labelling and information were compiled from Directive 98/79 of the European Community (EC), relevant World Health Organization (WHO) documents and studies on end-users' performance of RDTs. Typography and readability level (Flesch-Kincaid grade level) were assessed.

Results: Forty-two RDT kits from 22 manufacturers were assessed, 35 of which had evidence of good manufacturing practice according to available information (*i.e.* CE-label affixed or inclusion in the WHO list of ISO13485:2003 certified manufacturers). Shortcomings in devices were (i) insufficient place for writing sample identification (n = 40) and (ii) ambiguous labelling of the reading window (n = 6). Buffer vial labels were lacking essential information (n = 24) or were of poor quality (n = 16). Information inserts had elevated readability levels (median Flesch Kincaid grade 8.9, range 7.1 - 12.9) and user-unfriendly typography (median font size 8, range 5 - 10). Inadequacies included (i) no referral to biosafety (n = 18), (ii) critical differences between depicted and real devices (n = 8), (iii) figures with unrealistic colours (n = 4), (iv) incomplete information about RDT line interpretations (n = 31) and no data on test characteristics (n = 8). Other problems included (i) kit names that referred to *Plasmodium vivax* although targeting a pan-species *Plasmodium* antigen (n = 4), (ii) not stating the identity of the pan-species antigen (n = 2) and (iii) slight but numerous differences in names displayed on boxes, device packages and information inserts. Three CE labelled RDT kits produced outside the EC had no authorized representative affixed and the shape and relative dimensions of the CE symbol affixed did not comply with the Directive 98/79/EC. Overall, RDTs with evidence of GMP scored better compared to those without but inadequacies were observed in both groups.

Conclusion: Overall, malaria RDTs showed shortcomings in quality of construction, design and labelling of boxes, device packages, devices and buffers. Information inserts were difficult to read and lacked relevant information.

Background

The use of malaria RDTs is rapidly expanding

Prompt parasitological confirmation by microscopy or alternatively by RDTs is recommended in all patients suspected of malaria before treatment is started [1]. As a consequence, malaria rapid diagnostic tests (RDTs) are increasingly used as a diagnostic tool in both malaria endemic and non-endemic settings: in 2007, more than 70,000,000 tests were performed [2].

Malaria RDTs are so-called immunochromatographic tests that detect *Plasmodium* antigens in the blood by an antigen-antibody reaction on a nitrocellulose strip. The antigen-antibody complex is conjugated to colloidal gold, and a positive result is visible as a cherry- or purple-red coloured line. Apart from a control line, there are one, two or three test lines: the so-called two-band

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tests comprise a control line and a single test line, and are mostly designed to diagnose *Plasmodium falciparum*. Their targets are either histidine-rich protein-2 (HRP-2) or *P. falciparum*-specific parasite lactate dehydrogenase (Pf-pLDH). Three-band RDTs display a second test line mostly targeting antigens common to the four species such as pan-*Plasmodium*-specific parasite lactate dehydrogenase (pan-pLDH) or aldolase. The four-band RDTs have an additional third test line targeting *Plasmodium vivax*-specific pLDH (Pv-pLDH).

Written instructions add to the correct performance and interpretation of RDTs

RDTs are accurate and robust but they have limitations linked to design, production and distribution [3-8]. In addition, there are errors at the level of the end-user, which apply to both laboratory staff and field workers and are related to sampling, testing and interpretation of RDTs [9,10]. Clearly written instructions can add to the comprehensibility and maximize RDT kit performance [9,10]. On this basis, the World Health Organization (WHO) designed easy-to-read generic job aids [11].

During field visits in Africa, teams of the Institute of Tropical Medicine (ITM) occasionally noted shortcomings in RDT kit boxes, content and instructions. In addition, part of the interpretation errors that were observed during a recent external quality assessment (EQA) on RDTs were shown to be related to errors in the information inserts of the RDT kits used [4]. Inspection of these information inserts also revealed a large variety in layout and readability, as well as variations in the adequacy of labelling of RDT boxes and devices.

Objectives of the present study

In view of the observations above, it was decided (i) to assess malaria RDT kits for adequate and correct design, construction and labelling of boxes and components, and (ii) to study the readability and accuracy of their information inserts.

Methods

Selection of RDT kits

Malaria RDTs marketed as devices consisting of cassettes, cardboard boxes and hybrids (nitrocellulose strips to be dipped into plastic wells) were selected. They were checked for the presence of the CE label and evidence of good manufacturing practice (GMP) based on their inclusion in the WHO lists of RDT manufacturers and distributors complying with ISO13485:2003 or US FDA 21 CFR 820 production norms [12].

As this study was not intended to score RDTs individually, it was decided not to display the RDT brand and kit names, in line with previous comparative studies assessing RDTs [3-5,13].

Criteria used for RDT kit assessment and procedure

For packaging, design and labelling, assessment criteria were compiled from requirements listed in regulatory documents such as the Directive 98/79/EC and the European Community (EC) as well as relevant WHO documents [14-17]. Criteria for information inserts and device design from studies on end-users' performance and RDT instructions were pooled [5,7,9,10,18-24]. Inadequacies were defined as listed in Table 1.

RDT kit package, device package, device and buffer vial

The RDT kit packages were assessed for type (box versus plastic bag), material (simple and plasticized cardboard) and the presence and quality of the printed information. Information displayed on the package considered as essential included the RDT kits and manufacturer's names, expiry date, number of tests included, storage requirements and a reminder to read the instructions before use. A referral to the intended use of the RDT kit was looked for, either by the RDT kit name or by an additional text. The expiry date mentioned on the box was matched with those of the other RDT kit components. For CE labelled RDT kits produced by companies outside the European Economic Area, the affixing of the so-called authorized representative of the company in the EC (EC-REP) was assessed. Kits were assessed for sampling material needed included or not.

The package of the test device was checked for quality (humidity-proof material) and essential information including RDT kit name, lot number and expiry date. In addition, the desiccant was checked for composition, warning label and presence of colour indicator.

The RDT devices (cassette or cardboard housing the nitrocellulose strip) were assessed for clearness of design and construction including referral to the RDT kit's name. The space allocated for sample identification was evaluated for dimensions and ease of writing. A space of minimal 0.5 cm height and 4 cm wide was considered as adequate for handwriting of sample identification. The labelling of buffer wells, sample wells and reading windows including the places of appearance of the control and test lines (further referred to as reading label) were assessed for visibility and unambiguous interpretation.

The buffer vials were assessed for leak-proof closure, and their labels for quality of adherence and print. The information displayed on the label was assessed for the presence of RDT kit name, lot number, expiry date and storage conditions.

RDT information insert

RDT kits were checked for the presence of an information insert and a job aids (short procedure version), of which date of release and version number were assessed.

Table 1 Number of RDTs (n = 42) with inadequacies in malaria RDT boxes, device packages, devices, buffer vials and package inserts*

Items considered to be inadequate	Number (%)
Box: construction and design	
Materials: plastic bag or simple cardboard (not humidity-resistant)	9 (21.4)
No labels, no printed information or labels not humidity-resistant	6 (14.3)
Differences in name on device packaging, device, buffer and information insert	27 (64.3)
Box: information displayed	
No EC-REP mentioned on CE labelled RDTs, although required (n = 25)	3 (12.0)
RDT kit's name nor additional information refer to intended use	3 (7.1)
RDT kit's name incorrectly refers to *P. vivax* instead of non-*falciparum* species (n = 29)	4 (13.8)
Kit components not displayed	26 (62%)
Essential information lacking: expiry date, numbers of tests included, storage conditions	12 (28.6)
Kit contents:	
Capillary sampling system (lancet and alcohol swap) not included or not optionally included	24 (57%)
Blood transfer system (capillary, pipette or tube) not included	3 (7.1%)
Device package and content: construction and design	
Material not humidity-resistant	4 (9.5)
No desiccant or desiccant without saturation indicator	18 (42.9)
Device package and content: information displayed	
Essential information lacking: expiry date, lot number, test kit name	9 (21.4)
No warning label "do not swallow" on desiccant	6 (14.3)
Device: construction and design	
Space for sample identification too small or not writable with standard pen (felt pen needed)	40 (95.2)
No or incomplete RDT name on the device	29 (69.0)
No reading label or simultaneous presence two reading labels consisting of symbols only	6 (14.3)
Buffer: construction and design	
Buffer vial not leak proof	2 (4.8)
Label does not stick well to the vial, prints are not humidity-resistant (n = 40)	16 (40.0)
Buffer: information displayed	
Essential information lacking: expiry date, lot number, storage conditions, correct RDT kit's name (n = 41)	24 (58.5)
No instructions included on how to pierce the buffer vial dropper (n = 15)	5 (33.3)
Package insert: information	
Absence of date of release or version number	20 (47.6)
Package insert: content	
Identity of target antigens not clearly mentioned	2 (4.8)
No referral to biosafety precautions (gloves, safe waste disposal, etc.)	18 (42.9)
Major differences between depicted and real device (n = 40)	8 (20.0)
Use of figures with unrealistic colours (*e.g.* control and test lines depicted as green)	4 (9.5)
No data on test characteristics (sensitivity, specificity)	8 (19.0)

*Total number of RDT kits = 42 unless otherwise stated.

Layout and figures

The figures were counted and their dimensions measured. Their total surface area was calculated and expressed as a percentage of the total surface of the information insert. The figures were assessed for their concept (pure black and white versus use of colours) and conformity with the real devices.

Typography

The font size of the predominant letter type used (excluding the bibliography section) was measured in Cicero using a typometer (Rotring-werke Riepe KG, Hamburg, Germany) as the "kp" distance from the top of the highest ascender (top of the lower case letter k) to the bottom of the lowest descender (bottom of the lower case letter p). The opening of the characters was visually assessed for the characters "c, o and a", by covering them for their lower two-thirds and checking whether they were still correctly readable (open letter type) versus read as an "o" (closed letter type). The interline spacing was assessed by measuring in Cicero with a typometer the distance between the base line of two successive rows and then subtracting the font size.

Fonts of open letter types and interline spacing equal or larger than 2 are better readable compared to fonts of closed letter types and interline spacing smaller than 2, especially at larger text columns. For patient education materials and health instructions, font sizes of 12 or larger are recommended [25,26].

Readability level

For assessment of the readability level, the sessions about blood sampling, procedure and interpretation in the English text version were copied or retyped in Microsoft Word (Microsoft Corp., Redmond, WA, U.S.A.) and checked for correct spelling and syntax construction. Follow-up editing was performed as described elsewhere [27]. Next, the text fragments were copied into an on-line readability assessment tool, which generates different reading indices [28]. The Flesch-Kincaid grade level [29] was calculated. This grade-level expresses the U.S. grade-level equivalency of the skills required to read a particular document. For patient education materials and health related information, the recommended level is ≤ 6^{th} grade level [25,30].

Accuracy and relevance of information

The following items were actively looked for: description of the RDT test principle, target antigens, listing of required materials provided and not, description of sampling procedures and biosafety precautions. The RDT test procedures were studied with reference to common errors made by end-users in the field (Table 2) [5,9-11,18,19,23]. The interpretation section was assessed for the complete description of invalid results and *Plasmodium* species differentiation as well as for listing causes of false negative and false positive results. The description of test characteristics was assessed for mentioning the diagnostic accuracies related to the different *Plasmodium* species and parasite densities. Bibliographic references were checked for relevance with regard to RDT performance in general and information on the RDT kit's performance in particular.

Table 2 Number of RDT information inserts (n = 40) addressing critical steps in procedure and interpretation

Items addressed in procedure section	Number (%)
Bring the RDT device and buffer to room temperature	32 (80.0)
Check the integrity of the device package	9 (22.5)
Check expiry date	27 (67.5)
Use the device immediately after opening	28 (70.0)
Place the device on a level surface	0 (0.0)
Check the desiccant for signs of exposure to humidity	11 (27.5)
Write down sample identification	3 (7.5)
Wipe finger with alcohol	26 (65.0)
Allow the finger to dry before pricking	12 (30.0)
Hold the transfer device (loop, straw) vertical	8 (20.0)
Hold the buffer vial vertical	12 (30.0)
Do not to use another buffer than the one provided with the kit	9 (22.5)
Use an adequate light source for reading	3 (7.5)
Items addressed in interpretation section	**Number (%)**
All possible line combinations for invalid test results are mentioned	12 (30.0)
All possible test line combinations for positive test results are mentioned	31 (77.5)
Interpretation of a faint test line as positive is mentioned	8 (20.0)
Causes of false negative results are mentioned, in particular low parasite densities	11 (27.5)
Causes of false positive results are mentioned, *e.g.* presence of the rheumatoid factor	3 (7.5)
Persistence of HRP-2 is mentioned	19 (47.5)
To repeat the test in case of a negative RDT result and persistent suspicion of malaria is mentioned	1 (2.5)

Assessment, data registration and statistical analysis

Two observers trained in the use of RDTs independently assessed the RDTs according to the described criteria. Discrepant observations were discussed together with the other investigators and a consensus was reached. Data were registered in an Excel sheet (Microsoft Corp., Redmond, WA, U.S.A.).

Results

Panel of RDT kits

For the purpose of this study, 51 RDT kits were ordered at 29 companies. Seven companies (representing nine RDT kits) did not reply despite several reminders. The final panel consisted of 42 RDT kits from 22 companies. Nearly all (39/42, 93%) RDT kit formats were cassettes, further there were one cardboard and two hybrid kits. Two RDT kits consisted of individually wrapped RDT packages containing all materials for a single test (cassette, disinfectant, lancet and buffer). They will be further referred to as "Single RDT kits". Table 3 lists the RDTs according to their evidence of GMP. Table 1 lists the number of the RDTs with inadequacies in boxes, devices, buffer vials and information inserts.

RTD kit package, device and buffer vial

RDT kit package

Thirty-eight RDT kits arrived as cardboard boxes; four kits arrived in plastic bags. Two of these plastic bags contained a cardboard box to be folded by the end-user, resulting in a total of 40 boxes and two plastic bags as the package of use on the bench. All but one box displayed an indication in the RDT kit's name or in the test description that the RDT kit was intended for malaria diagnosis. The two plastic bags did not display

Table 3 Overview of the RDT kits evaluated in the present study

RDT format	*Plasmodium* antigens targeted	Number	Evidence of GMP		
			CE mark	WHO list[†]	Total
Two band	HRP-2	7	4	5	7
	pan-pLDH	1	1	0	1
	Pv-pLDH	1	1	0	1
Three band	HRP-2, pan-pLDH	11	5	7	9
	HRP-2, aldolase[‡]	5	5*	5	5
	HRP-2, Pv-pLDH	4	1	1	2
	Pf-pLDH, pan-pLDH	6	5	6	6
Four band	HRP-2, Pv-pLDH, pan-pLDH	7	3	5	6

*One three-band test (HRP-2, aldolase) is FDA approved.

[†]WHO list of ISO:13485:2003 certified manufacturers and their RDT products (list of known commercially available antigen detecting malaria RDTs) [12].

[‡]One of these RDTs used both aldolase and pan-pLDH as pan-malarial antigen.

any information. A company name was listed on all the 40 boxes, but for 12 kits, it was not clearly mentioned whether this name represented the manufacturer or the distributor.

The lot number and expiry date were listed on all boxes. One of the Single RDT kits showed both lot number and expiry date on the outer box containing the single packages, but not on the single packages themselves. There were no discrepancies between the expiry dates on the RDT kit box and those of the contents except for two buffer vials with expiry dates extending those printed on the RDT kit box.

The number of tests included and a reminder to read the instructions before use were not displayed on four and ten boxes respectively. All the 40 boxes showed information on storage temperature requirements, by written text, symbols or both. Apart from a single symbol, *i.e.* a penguin expressing "do not freeze", all symbols were internationally recognized symbols complying with EN 980:2008 or FDA 2004, 21 CFR 809.10 and 21 CFR Parts 610 and 660. Capillary blood sampling systems were included in eight kits and proposed as optional in ten other kits. Blood transfer systems were missing in three kits.

Device packages

Four of 42 device packages were not made of humidity-resistant material. Most of the packages were easy to open by tearing a pre-cut lid of the package. However, for three packages, scissors had to be used to open the packages properly.

A desiccant was present in all but one package. Three of the 41 desiccants did not show a warning that the desiccant was harmful: two of them (from one manufacturer) were tablets looking like drug pills. Seventeen desiccants (including the two tablets) had no colour indicator of humidity saturation.

RDT devices

Space for writing was too small in 40 of the 42 devices (Figure 1). For two cassettes, a felt pen was required as a standard pen failed to mark.

Most cassettes (35/39) had separate wells for sample and buffer application, four had a single well for sample and buffer application. There was no uniform labelling of the wells: for instance characters "S" and "A" were used randomly for the sample well, buffer well and combined sample/buffer well (Figure 2). Fifteen cassettes showed at the distal end a window or holes that might be confused with a sample or buffer well (Figures 1 and 2).

The reading label was indicated with acronyms (n = 20), characters or numbers. Acronyms included abbreviations such as "Pf" or "pan", they were printed on the plastic housing or on a label and were well readable. Characters such as "C" (control line) and "T" and numbers were embedded in the plastic housings and were more difficult to distinguish (Figure 2). In one cassette, characters were printed on a label, which was not well fixed (Figure 1). In 14 cassettes, two labels were displayed at either side of the reading window (Figure 2), and one three-band cassette had no reading label at all (Figure 2).

RDT buffer vials

The two Single kit RDTs contained a small buffer plastic ampoule in each device package, which were too small to display information. Fifteen buffer vials required clockwise tightening the vial cap to pierce the dropper vial nozzle, but for five of them, this was not mentioned in the information insert. For 13 vials, the label was not well fixed and the printed information on three of these labels was not humidity-resistant. Lot number and expiry date were not listed on five vials and storage conditions were missing on 10 vials.

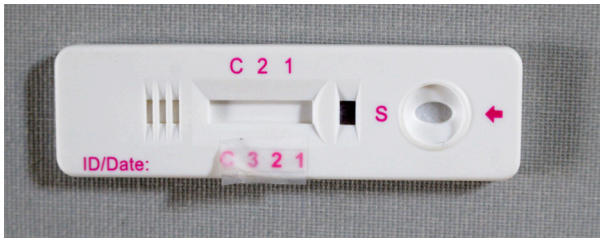

Figure 1 Four-band RDT. The allocated place for writing sample identification is too small. The grid at the left hand may be confused with a sample well. There are two different reading labels at each side of the reading window, of which the lower one is printed on a label that is not well fixed.

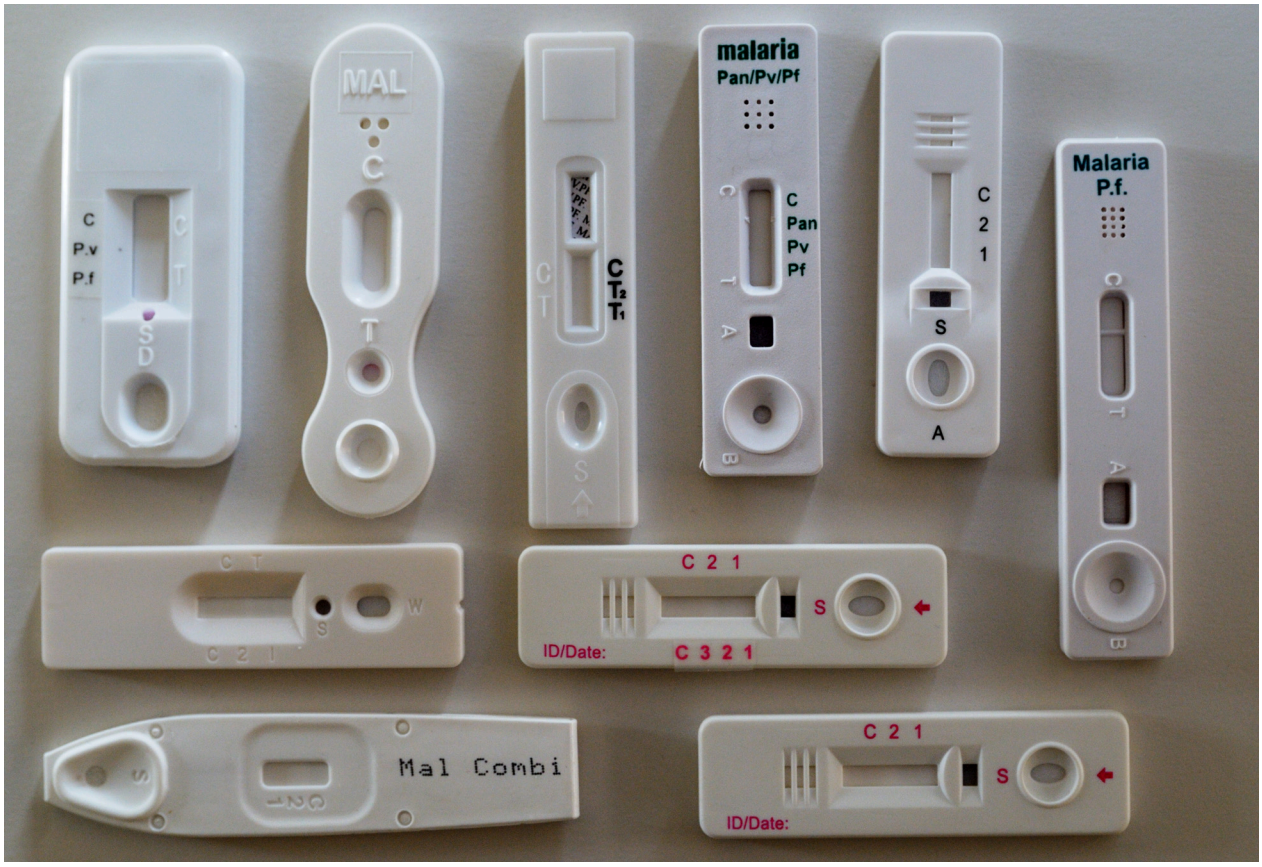

Figure 2 Example of RDT cassettes. Most of the cassettes have separated wells for sample and buffer application. There is no uniform labelling of the wells: different characters (*e.g.* "S", "A") are used randomly for the sample well, buffer well and combined sample/buffer well. The reading labels are indicated with acronyms, characters or numbers.

RDT information insert

The information inserts of the two Single RDT kits were not considered: one of them contained a simple job aids explaining the procedure by figures only, the other contained a shortened version of the information insert of the same RDT marketed as laboratory kit. All of the remaining 40 RDT kits contained an information insert of which seven had an additional job aids. Either version number or date of issue was missing in 11 and 13 of them; in five, both were missing.

Layout and figures

All 40 inserts included figures. The median number of figures per information insert was 8.5 (range 2 - 25) and figures accounted for a median surface ratio of 7.2% (range 0.4% - 33%) of the entire insert. The median size of the figures was 2.4 × 2.0 cm, the smallest and largest figure measured respectively 1.0 × 0.3 cm and 6.7 × 8.0 cm.

All inserts used figures to illustrate the interpretation section. Other figures depicted blood sampling (n = 17), application of sample and buffer (n = 21) and a clock indicating the correct reading delay (n = 10). Fourteen inserts used red colour to indicate control and test lines but in four, they were pictured green or blue. Most inserts (n = 35) showed differences between depicted and real devices of which some were major, such as discrepancies between characters used for sample and buffer well identification (n = 4) and differences in labelling of the reading window (n = 5) (Figures 3 and 4). One insert mentioned a reading delay of 15 minutes, but the illustration mentioned a reading delay of 20 minutes (Figure 5).

Typographic features and readability

Figure 6 displays the font size and line spacing of the information inserts. Median font size was 8 ± 1.3, none of them exceeded 10. User-unfriendly typographic features included combinations of font sizes of eight or smaller with a closed letter type (n = 10) or with line spacing lower than two and more than 12 words per line (n = 14) (Figure 7). Median readability level was grade 8.9 (range 7.1 - 12.9) and 18 and four of the inserts' readability levels were above grade 9 and 10 respectively (Figure 8). Readability levels of the job

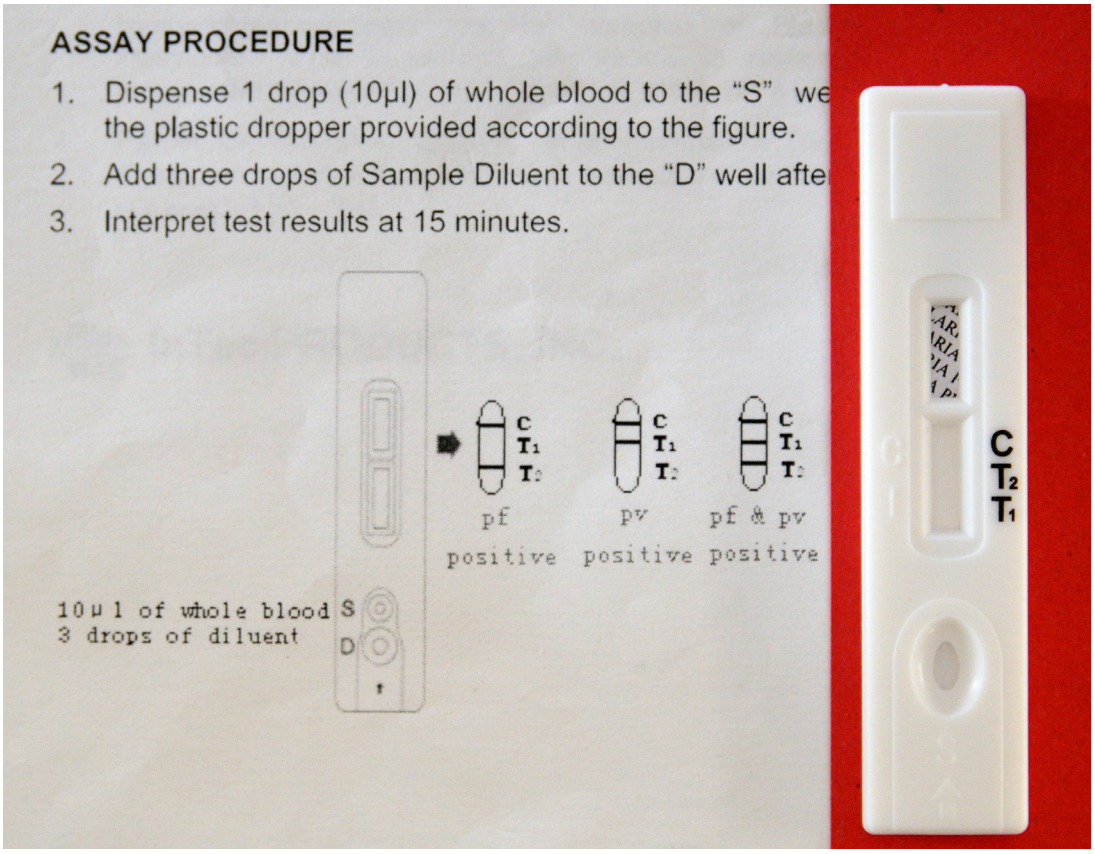

Figure 3 Interpretation section of the information insert and cassette of a *Plasmodium falciparum/Plasmodium vivax* RDT. The real device has a single sample/buffer whereas the depicted one displays separate wells. The characters used for the reading label on the illustration are inverted compared to the real device.

aids were also high (median 8.5, range 5.1 - 9.4), and six out of seven exceeded the readability level of the most recent WHO job aids. Five inserts showed prints of very poor quality hindering reading of the text (Figure 9).

Accuracy and relevance of information

The RDT kit's principle was described in all 40 information inserts. All but two inserts mentioned the materials provided in the RDT kit, and seven inserts provided a complete list of the materials required.

All RDT kit inserts mentioned the required specimen (in all cases both capillary and venous blood), all but one mentioned the anticoagulant to be used. Capillary blood sampling through finger prick was described in 35 inserts, of which one also added sampling by venipuncture. By contrast, the heel prick was not described in any information insert.

Biosafety precautions included the use of gloves (depicted or mentioned in 21 inserts, Figure 10) and safe waste disposal (addressed in 16 inserts), but 18 inserts did not mention any information on biosafety.

From Table 2 it is clear that a number of critical steps in RDT procedures were addressed by only part of the RDT inserts. Among them, there were relevant steps such as writing down sample identification, correct positioning of the transfer and buffer vial and the need for an adequate light source.

The complete array of all control and test line combinations was listed by only nine inserts. Fourteen inserts mentioned the absence of all lines as an invalid result but not the presence of a test line in the absence of a control line (Figure 9). In addition, errors in the interpretation of test lines were observed. For instance, the combination of a Pf-specific and a pan-specific test line was interpreted as *P. falciparum* without mentioning the possibility of a mixed infection (12/34 three- and four-band RDTs). Likewise, the combination of a Pv-specific and a pan-specific test line in case of a four-band RDT was interpreted as a *Plasmodium vivax* infection without mentioning the possibility of a mixed infection with *Plasmodium ovale* and *Plasmodium malariae* (6/7 RDTs). In addition, a visible pan-pLDH line was

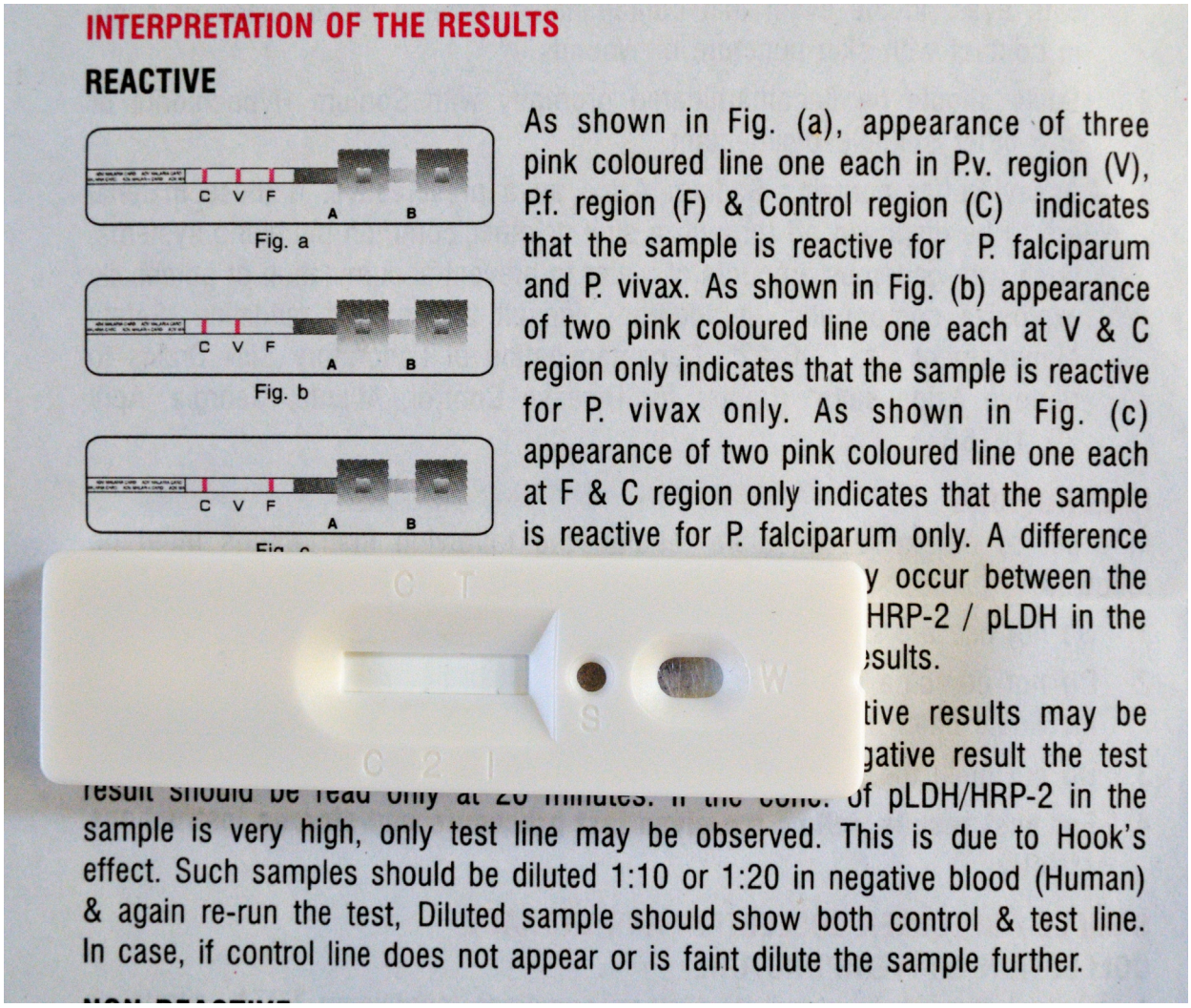

Figure 4 Interpretation section of the information insert and cassette of a *P. falciparum/P. vivax* RDT. Shape and labels of wells and reading window are different between the real and the depicted device. Characters are embedded in the plastic housing and poorly discernable. The text is correct and complete (even the prozone effect and how to deal with it) but less readable (Flesch-Kincaid grade level 9.1).

interpreted as a *P. vivax* infection (instead of non-*falciparum* species) in two inserts.

Few inserts mentioned causes of false positive and false negative results (Table 2). One insert recommended to repeat the test in case of a negative RDT result and persistent suspicion of malaria, another warned about the prozone effect as a cause of a false negative result (Figure 4). RDT test results during treatment follow-up were addressed in 13 inserts, but the information listed in nine inserts was presented in a scattered way and only one insert clearly mentioned that HRP-2 persistence does not indicate a failed therapeutic response.

RDT test characteristics

Eight inserts did not provide information on sensitivity or specificity. Diagnostic characteristics were mostly expressed for *P. falciparum* and *P. vivax* (n = 31 and n = 22 respectively), only one insert mentioned test characteristics for *P. ovale* and *P. malariae*. Sensitivity for *P. falciparum* was expressed by parasite density range in 10 inserts.

Bibliography cited in the information inserts

In total, 45 different references were used in the bibliography of the information inserts. One third of them referred to the original description of the target antigens, another 12 referred to general information on malaria and its diagnosis. Thirteen inserts cited evaluation studies of RDTs, but only three RDT kits referred to product-related studies. Two panels of identical references were shared by nine and eight inserts respectively.

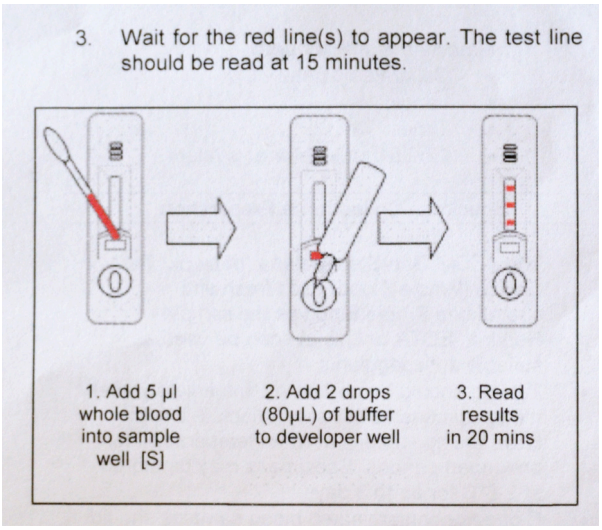

Figure 5 Information insert of a three-band RDT, procedure section. There is a discrepancy between the reading time mentioned in the text compared to that showed on the illustration.

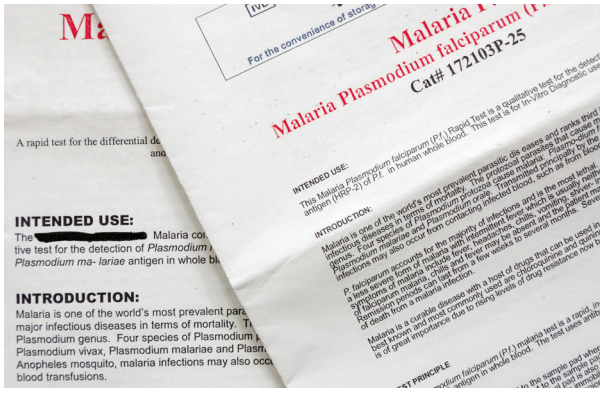

Figure 7 Example of typography used in RDT information inserts: the package insert on the top is user-unfriendly (font size 6, line spacing 0,5, close letter type, average number of words per line 21, Flesch-Kincaid grade 9,5). The package insert on the background, from the same company but for another RDT uses a better typography (font size 8, line spacing 2, open letter type, average number of words per line 14), but the readability is still elevated (Flesch-Kincaid grade 9,8).

RDT kits' names

Inconsistencies in the RDT kit names and referrals to target antigens were observed. For instance, four RDT kits had names referring to *P. vivax* although they used a pan-species *Plasmodium* antigen. Two other inserts did not mention the identity of the pan-species antigen (aldolase versus pan-pLDH). Five RDT kits from one manufacturer were supplied in identical boxes, carrying the same names and identical prints. Furthermore, there were slight but numerous differences between names as displayed on boxes versus those noted on device packages (eight had no brand name affixed), devices (15 differences) and information inserts (eight differences). Similar observations were made for buffer vials: five vials displayed only the manufacturer's name and one vial did not show brand nor manufacturer's name.

RDT kits' duplicates

During assessment of the RDT kits, apparent similarities between different RDT brands were observed. These similarities concerned, amongst others, design and shape of the device and content and layout of the information insert (*e.g.* numbers of samples used for calculation of test characteristics). In that way, six products were assumed to represent a common design and production platform for 16 different RDT brands.

Relation with CE marking and WHO listing

Overall, RDTs with evidence of GMP (n = 35) scored better compared to those without (n = 5), although inadequacies, errors and omissions were observed between both groups. Three CE labelled RDT kits produced outside European Economic Area (EEA) had no EC-REP indicated neither on the box nor in the information insert. For these kits, the CE symbol as displayed

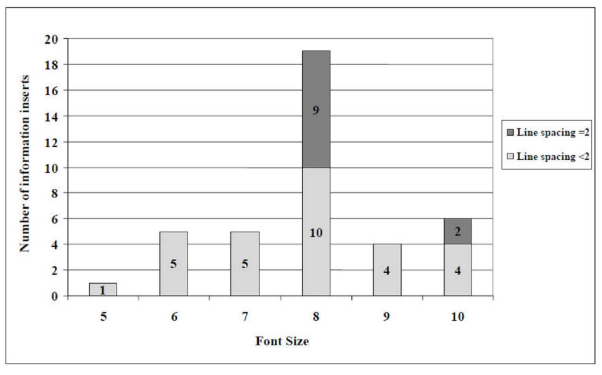

Figure 6 Typographic features of RDT information inserts (n = 40): font sizes and line spacing.

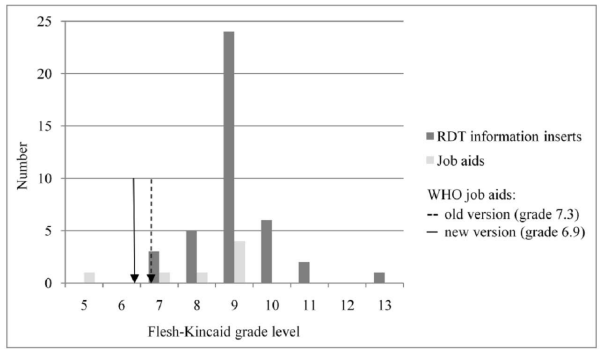

Figure 8 Readability of the RDT information inserts (n = 40) and job aids (n = 7) expressed as Flesch-Kincaid grade level.

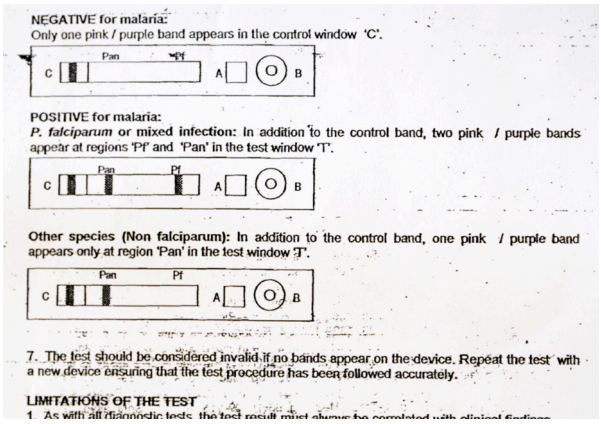

Figure 9 Interpretation section of the information insert of a three-band *P. falciparum*/pan-species RDT. For invalid tests, only the absence of all lines is mentioned, not the possibility of a visible test line without a control line. The invalid results are not depicted. The print quality is poor, no colours are used for the control and test lines.

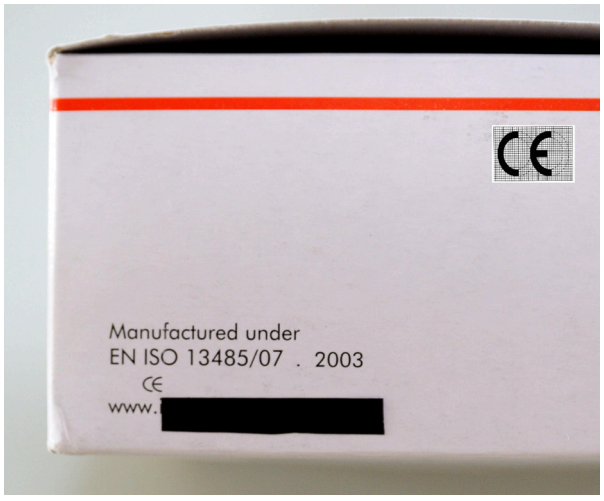

Figure 11 CE-label displayed on a RDT kit box. The shape and relative dimensions of the characters do not comply with the requirements as mentioned in the EC Directive 98/79 (depicted in the insert, upper right corner). There is no authorized representative (EC-REP) affixed, although required.

on the package had not the shape and relative dimensions of the 98/79/EC directive (Figure 11). Six of the eight RDT kits that did not mention data on sensitivity and specificity were CE labelled.

Discussion

Previous studies demonstrated that RDT manufacturers' instructions are insufficient to ensure accurate test performance by community health care workers, and well-designed instructions such as the WHO generic job aids have proven to increase performance [9,10,19,31]. In practice however, such job aids still need to be adapted to the particular RDT brand used on site and, depending on the chains of supply, different RDT brands and versions may be available. For market exploration and choice of RDTs, laboratory managers will orient to RDT kits' names and labelling. Clear design and labelling of RDT kit components will contribute to correct storage and use; and laboratory staff will rely on RDT inserts for background information, adaptations of the generic procedures, interpretation and trouble-shooting.

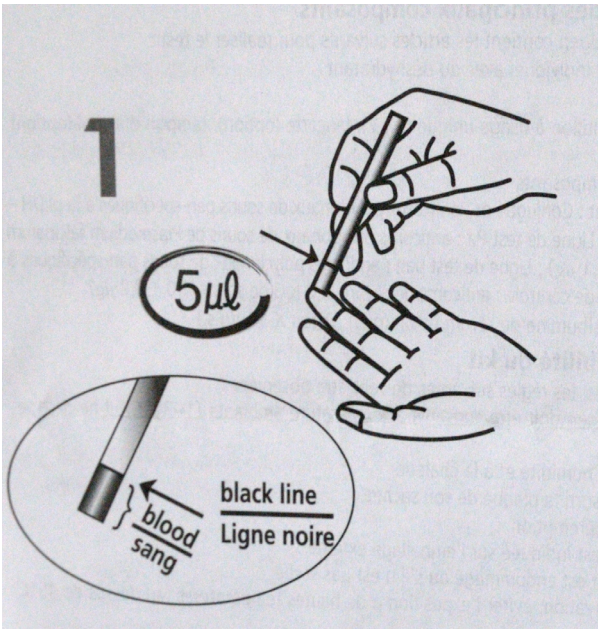

Figure 10 Illustration depicting sampling of capillary blood. The health care worker's hand is depicted without gloves. The simultaneous presence of English and French text may be difficult for a non-experienced reader.

The design of the present study has its limitations. For instance, mainly RDTs marketed as cassettes were considered. However, cassettes are the mostly used platform and preferred by end-users over the strip format [9]. Besides, only a part of the marketed brands was evaluated; however, this evaluation studied about half of the 80 brands worldwide-marketed [2] including those frequently used in endemic and non-endemic settings.

With regard to the assessment of the information inserts, it should further be noted that the Flesch-Kincaid label (as discussed below) is only a proxy measure of readability. In addition, the layout of the inserts was assessed for typography but not for other features such as adequate use of headings, bullets, boldfacing, and amount of white space [32,33]. Finally, although RDTs were presently assessed against compiled criteria based on relevant documents, they were not evaluated by end-users in a real-life setting. On the other hand, as far as

known, this is the first time that in vitro diagnostic medical devices (IVDs) were assessed for these characteristics.

Problems in design and labelling of the RDT kits' components

Despite these limitations, much information was generated on the quality and appropriateness of RDT packaging, labels and inserts. Apart from two exceptions (the plastic bags), boxes were well labelled, but shortcomings in labelling of device packages and buffer vials were more numerous. The absence of blood sampling and transfer system which was observed in nearly a quarter of RDT kits may create logistical problems when used in field settings. The problems in device design were of most concern. Characters indicating wells and reading labels that were embedded in the plastic cassette housings are difficult to distinguish and the simultaneous presence of two reading labels may cause confusion. Standardized and unequivocal characters or acronyms should be used for designating wells and reading labels and clear labelling with contrasting print should be ensured.

Some design issues were not compiled from previous studies but originated from ITM observations. Some of them may look trivial but have consequences in daily practice. For instance, device packages without pre-cut lids require scissors to open, which is neither safe nor practical in busy and remote settings. The space allocated for sample identification on most cassettes was large enough for writing down a sample number but not a patient name: this may meet the requirements in computerized settings but not those in a non-computerized field setting, where full names are written as recommended by WHO job aids instructions [16]. Likewise, it is evident that use of a felt pen for writing down sample identification is inappropriate for a field setting. Another example was the clockwise tightening of the buffer vial's cap for piercing the dropper bottle nozzle: ITM teams observed that laboratory staff unaware of this procedure simply cut off the distal end from the nozzle. The resulting opening tended to be very wide resulting in a too large volume of buffer added to each test and early emptying of the buffer vial. The RDT cassettes that remained without buffer were run with other buffers or with injection water, at the risk of causing false positive results [5].

Readability level and typography of the information insert

The Flesch-Kincaid grade level used in the present study has been demonstrated to be reliable and valid and is frequently used in health care issues such as consumer medical information (CMI) and patient education materials [27,30]. Its use in the present context should be interpreted with caution. Like any other reading formula, the Flesch-Kincaid readability tool assesses text structure but does not take into account the content. It further refers only to US grade levels and applies to English language. Other factors such as motivation and previous experience may influence comprehension, and linguistic and cultural issues may interfere [33]. Despite these limitations, it is of note that readability levels of all inserts exceeded the 6^{th} grade level, while the recommended level for health related information is equal or lower than this level [27,30]. The readability level of the job aids scored slightly better, but still higher compared to the WHO generic job aids. Too elevated reading levels have been consistently demonstrated in CMI documents such as those of home pregnancy tests, blood glucose monitoring and home blood pressure monitor equipment as well as in patient education brochures [25-27,32,34,35]. It should also be taken into account that end-users in endemic settings are likely to be non-native speakers of the language of the information insert (*e.g.* English, French, Portuguese), and may operate in stressful situations such as environmental disasters and war [36,37] which decrease actual reading levels [38]. Apart from the bad quality prints and small figures, the observed user-unfriendly typographic features may add to the decreased readability of the inserts, which were consistent with those documented for CMI materials [25,30,32,34,35].

Content of the information insert

The lack of referral to biosafety procedures in nearly half of the inserts was striking and unacceptable, in particular because this is clearly mentioned in the WHO generic job aids [11]. Likewise were the differences between depicted and real devices and the use of non-realistic colours for depicting test lines which do not comply with WHO recommendations [16]. Numbers and sizes of illustrations did not comply with the established standards for patient education materials and CMI [25,35,39].

The shortcomings in the RDT test interpretation session were in line with observations made during a recent external quality assessment on RDTs in a non-endemic setting. During that session, not reporting a mixed infection in case of the simultaneous presence of *P. falciparum*- and pan-specific test lines was demonstrated to be linked to the information inserts of the RDT kits used [18]. Although extremely rare in the experience at ITM, the presence of a visible test line in the absence of a control line points to invalid test results and should be added to the spectrum of possible line combinations, preferably with a picture. In addition, the persistence of HRP-2 after successful treatment and the production of pLDH by gametocytes should be clearly mentioned.

The poor description of diagnostic characteristics in the information inserts was another concern. Although current directives and recommendations do not specify details about origin, numbers and statistical validity of these test characteristics, manufacturers should be encouraged to provide as detailed and sound data as possible, including data on sensitivity in relation to parasite density and *Plasmodium* species. The low diagnostic sensitivity for *P. malariae* and *P. ovale* is well known [40]: few studies have included enough samples to provide reliable data for both species. Those that did mostly found a poor sensitivity, in particular for *P. malariae* [40-44]. In the absence of a thorough evaluation for both species, one could consider adding a statement mentioning the low overall diagnostic sensitivity for both species to the information insert, in order to avoid unrealistic expectations by the end-user relying on the pan-*Plasmodium* species nature of the targeted antigen [40].

The cited bibliography mainly referred to the original papers on the description of the antigens or general RDT evaluations. In addition to references addressing the RDT kit itself, references to one or more of the recent reviews on RDTs or WHO/FIND documents could be added, as they contain relevant information on the use and limitations of RDTs.

Names and duplicates of RDTs, relation to GMP and CE labelling

Among the inadequacies, erratic and inconsistent names were a frequent finding: they ranged from minor differences in RDT brand names as displayed on boxes, devices and their packages and information inserts to brand names suggesting *P. vivax* despite using a pan-pLDH target. In addition, shortcomings with regard to clear specification of target antigens were noted. For a laboratory manager finding his way among many other diagnostics and supplies, it is essential to get a quick and reliable idea about the intended use and the target antigens of RDTs. An unequivocal code for naming and short test descriptions should be considered, with mentioning of the (abbreviated) antigens as a requisite (*e.g.* Pf-pLDH, HRP-2 etc.).

With regard to the presumed RDT kit duplicates (kits presenting with similar presentation suggesting a shared design and production), it should be noted that WHO and FIND recognize this phenomenon [45]. WHO defines so-called "re-branded" products as products manufactured under identical conditions at the same manufacturing site as the original product, but labelled with a different product name and identifier. WHO encourages, in such case, joint application for the prequalification program or test evaluations [45-47]. The CE recognizes also the "re-branding" for commercial interests. The coexistence of multiple names for the same product however may create difficulties for instance in retrieving published information on test evaluations and may add to the complexity of post marketing surveillance, including traceability in case of batch recalls. To prevent these problems, the requirement of "re-branded" RDT kits to mention the original manufacturer should be considered. In addition, any RDT kit label (whether original or re-branded) should clearly distinguish the names of the manufacturer from that of the local distributor.

Shortcomings and errors were observed among CE-labelled and WHO-listed RDTs. Not affixing the EC-REP when required and not mentioning information on RDT test characteristics do not conform the 98/79/EC Directive [48]. It should further be noted that in case of malaria RDTs, the CE-label by itself is not a guarantee for intrinsic quality of performance. The 98/79/EC Directive includes the "Annex II", which lists diagnostics for which market release of any new lot has to be preceded by testing and approval by a competent authority, the so-called notified body. For diagnostics that are not listed in the "Annex II" (such as malaria RDTs), such testing and authorization are not required. Acquisition of the CE-label for these diagnostics is a purely administrative process, in which the manufacturer himself draws up the EC declaration of conformity. Unfortunately, the majority of laboratory and medical staff are unaware of this procedure. This can create a sense of "over-confidence" in CE-labelled products, based on the perception of quality associated with European labels. The inclusion of RDTs for malaria and other tropical diseases in the "Annex II" could represent a significant support for countries with weak regulatory overview.

What can be done to improve the quality of RDT package and information inserts?

Many shortcomings such as incomplete and incorrect labelling of boxes, device packages, cassettes and buffer vials can be easily remediated at minimal costs. End-users and manufactures should reach consensus on uniform codes for labelling wells and reading windows and gradually reinforce the requirements for inserts and packaging. Generic recommendations as how to layout and how to appropriately design information inserts as well as use of figures can be found in the literature on CMI [25,35,39], RDT specific guidelines have been issued by the WHO [16]. Readability, cultural and linguistic backgrounds should be taken into account [23], and all texts and figures should be assessed for appropriateness and comprehension among the targeted end-users [10,49]. For the content of RDTs, the reference documents that were used to compile Tables 1 and 2 can give guidance. Examples of such checklists are added as additional files (Additional file 1 Table S1 and Additional file 2 Table S2). The information inserts

should highlight key points in performance and interpretation in order to reduce the likelihood of potential errors.

With regard to user-friendliness and adequacy of RDT presentation and instructions, interesting features not listed on the compiled criteria were noted. For instance, some device packages carried short instructions for use based upon the generic WHO job aids [16], witch may increase the test performance [9,10,19,31]. The availability of job aids for the different brands on the company websites in an adaptable text format may help Malaria National Programs to translate it in the end-user language and to adapt it on the local context. In addition, some RDT kits provided a glossary with explanation of the affixed symbols, which may help in their comprehension and acquisition and another five had all essential information printed on a single (lateral) side of the box, contributing to easy storage (Figure 12). Another asset was the presence of more than one buffer vial per RDT box, as shortage and replacement of buffer vials is a common problem in resource limited settings [5]. Likewise, the above described observations and corrective measures could be extended to other IVDs, such as human immunodeficiency virus RDTs.

Of course, it should be noted that adequate packages and information inserts by themselves are not a guarantee for competent use of RDTs. Simply distributing the RDTs and instructions does not work, and RDT instructions on their own will not change professional behaviours [20]. Thorough training and performance monitoring are needed for correct performance [16].

Conclusion

In conclusion, malaria RDTs showed shortcomings with regard to quality of construction, design and labelling of boxes, device packages, devices and buffers. Information inserts were difficult to read and lacked relevant information. Particular problems were observed in the consistency and appropriateness of RDT brand names and in the referral to the antigens used. In general, CE-labelled and WHO-listed RDTs scored better compared to those without but inadequacies were observed among these RDTs. Addressing the quality of RTD package and information inserts in evaluation programs such as the WHO/FIND products testing program could stimulate the manufacturers to remediate these shortcomings. Likewise, inclusion of malaria RDTs in the "Annex" II of the 98/79/EC directive might represent a powerful support from the European Community towards the quality of *in-vitro* diagnostics in tropical countries.

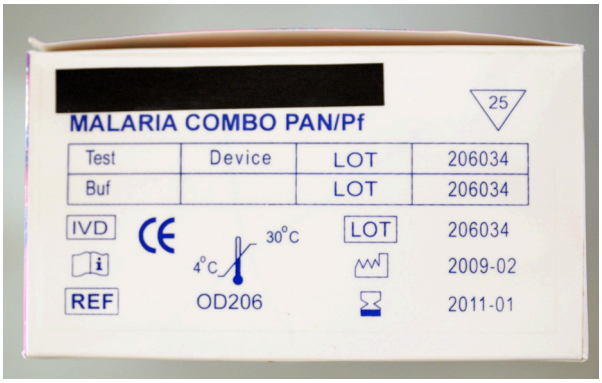

Figure 12 Example of the lateral side of a RDT box: all essential information is printed on a single side of the box, contributing to clear storage.

Additional material

Additional file 1: Example of operational checklist for packaging, labelling and instructions of RDTs.

Additional file 2: Example of a checklist for the content of the information inserts.

List of abbreviations

CE: Conformité Européenne; CMI: Consumer medical information; EC-REP: European Authorized Representative; EEA: European Economic Area; FDA: Food and drug administration; FIND: Foundation for Innovative New Diagnostics; FHML: Faculty of Health, Medicine and Life Sciences; GMP: Good manufacturing practice; HRP-2: Histidine-rich protein 2; ISO: International organization for standardization; ITM: Institute of Tropical Medicine; IVDs: *in vitro* diagnostic medical devices; *P: Plasmodium*; pan-pLDH: pan *Plasmodium*-specific parasite lactate dehydrogenase; Pf-pLDH: *Plasmodium falciparum*-specific parasite lactate dehydrogenase; pLDH: parasite lactate dehydrogenase; Pv-pLDH: *Plasmodium vivax*-specific parasite lactate dehydrogenase; RDT(s): Rapid diagnostic test(s); WHO: World Health Organization.

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Authors' contributions

PG and JM equally contributed to the present study and share first authorship. PG JM and JJ designed the study protocol, RR made substantial contributions to the concept and design of the study. PG, JM and VH carried out the test evaluations. PG, JM, CB and JJ analysed and interpret the results and drafted the manuscript. All authors critically reviewed the manuscript and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Chapter 5. RDTs for self-use by travellers

Self-diagnosis of malaria by travelers and expatriates: assessment of malaria rapid diagnostic tests available on the internet.

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Self-Diagnosis of Malaria by Travelers and Expatriates: Assessment of Malaria Rapid Diagnostic Tests Available on the Internet

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Abstract

Introduction: In the past malaria rapid diagnostic tests (RDTs) for self-diagnosis by travelers were considered suboptimal due to poor performance. Nowadays RDTs for self-diagnosis are marketed and available through the internet. The present study assessed RDT products marketed for self-diagnosis for diagnostic accuracy and quality of labeling, content and instructions for use (IFU).

Methods: Diagnostic accuracy of eight RDT products was assessed with a panel of stored whole blood samples comprising the four *Plasmodium* species (n = 90) as well as *Plasmodium* negative samples (n = 10). IFUs were assessed for quality of description of procedure and interpretation and for lay-out and readability level. Errors in packaging and content were recorded.

Results: Two products gave false-positive test lines in 70% and 80% of *Plasmodium* negative samples, precluding their use. Of the remaining products, 4/6 had good to excellent sensitivity for the diagnosis of *Plasmodium falciparum* (98.2%–100.0%) and *Plasmodium vivax* (93.3%–100.0%). Sensitivity for *Plasmodium ovale* and *Plasmodium malariae* diagnosis was poor (6.7%–80.0%). All but one product yielded false-positive test lines after reading beyond the recommended reading time. Problems with labeling (not specifying target antigens (n = 3), and content (desiccant with no humidity indicator (n = 6)) were observed. IFUs had major shortcomings in description of test procedure and interpretation, poor readability and lay-out and user-unfriendly typography. Strategic issues (*e.g.* the need for repeat testing and reasons for false-negative tests) were not addressed in any of the IFUs.

Conclusion: Diagnostic accuracy of RDTs for self-diagnosis was variable, with only 4/8 RDT products being reliable for the diagnosis of *P. falciparum* and *P. vivax,* and none for *P. ovale* and *P. malariae*. RDTs for self-diagnosis need improvements in IFUs (content and user-friendliness), labeling and content before they can be considered for self-diagnosis by the traveler.

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Introduction

In the nineties, malaria rapid diagnostic tests (RDTs) were suggested for self-diagnosis by travelers [1]. The idea was however abandoned after several studies had shown poor test performance and difficulties in interpretation of test results by the ill traveler [1–4]. Since then, major progresses have been made in RDT design and performance. Most of the currently marketed RDTs are so-called one-step RDTs that are more simple and user-friendly [5] compared to the multi-step RDT products evaluated in previous studies. Malaria RDTs are now easy to use handheld cassettes detecting antigens produced by the *Plasmodium* parasite which become visible as colored (mostly red) test lines within 20 minutes [6].

Recently, the decline in malaria burden in many parts of the world has made stand-by emergency treatment (SBET) more attractive for many travelers than the classic chemoprophylaxis. In the SBET strategy, travelers and expatriates to low-resource endemic settings carry an emergency malaria treatment (with reliable activity against *P. falciparum*) for self-administration when no medical attention is rapidly available. This option may be considered where the risk of adverse reaction to malaria chemoprophylaxis outweighs the risk of malaria infection [7,8] and is increasingly promoted by some experts in travel medicine [9]. Self-diagnosis of febrile illness with reliable malaria RDTs could accelerate early therapy (with the standby treatment), preventing complications and death, or avoid unnecessary use of antimalarials [10,11].

Nowadays, RDTs for malaria self-diagnosis are available through the internet, but their diagnostic accuracy and ease of use have not yet been studied. Also, the quality of instructions for

use (IFU) – which assure correct performance and interpretation of the RDT results [4] - can vary widely [12]. Therefore, the present study assessed both diagnostic accuracy and quality of packaging, labeling and IFU of RDT products marketed for self-diagnosis by travelers.

Methods

Ethics Statement

The study was approved by the Institutional Review Board of ITM and by the Ethical Committee of Antwerp University, Belgium. RDTs were performed on stored blood samples (''leftovers'') obtained as part of routine diagnostic work-up in international travelers suspected of malaria. In view of the absence of risk and the anonymous data processing, IRB deemed informed consent obsolete.

Study Design

RDT products for self-diagnosis were evaluated for diagnostic accuracy against a panel of 100 stored blood samples obtained in travelers suspected of malaria and for the quality of packaging and their IFU.

Patients and Samples

A total of 100 stored EDTA blood samples (including the four human *Plasmodium* species as well as malaria negative samples, Table 1) obtained from returned international travelers clinically suspected of malaria were selected. Samples were collected between October 2007 and September 2011 and were stored at $-70^{\circ}C$ at the Institute of Tropical Medicine (ITM), Antwerp, Belgium. Species identification was done by expert microscopy, corrected by four-primer real-time PCR [13].

Malaria Rapid Diagnostic Tests

Availability of RDT products for self-diagnosis on the internet was assessed using the search engine 'Google'. The following terms were used in both English and French: 'Malaria self-diagnosis', 'Malaria self-test', 'Malaria survival kit', 'Malaria home testing', 'Malaria autotest'. Three times a search was performed (January, April and June 2011). In addition, two manufacturers were contacted directly for availability of RDT products marketed for self-diagnosis (Standard Diagnostics and Access Bio Inc.). Another manufacturer (TODA PHARMA) had contacted himself ITM to inform that he had RDTs available for self-diagnosis.

Test Procedures

Tests were performed according to the manufacturer's instructions, except that a transfer pipette (Finnpipette, Helsinki, Finland) was used instead of the transfer device supplied with the RDT products. The first observer was the one who performed the test and read test results within the specified reading time. The second observer read test results within 5 additional minutes and was blinded to the results of the first observer. Both observers were blinded to the results of microscopy and PCR. The results of the first observer were considered and compared to the second observer to determine interobserver agreement. Test lines were scored for line intensity as described previously [14]. Faint intensity implies a barely visible test line which risks being interpreted as negative. In case of absence of the control line, the result was invalid and considered negative because it was assumed that travelers will not always have a second RDT available. In order to assess false positive results occurring upon reading beyond the recommended reading time (so-called ''back-flow phenomenon'' [15], the first observer scored test lines again at the end of the day, between 2 and 8 hours after initial reading.

RDT Packaging and Instructions for Use

RDTs' packaging and instructions for use (IFU) were assessed using a checklist adapted from a previous study [12]. In addition, typography, lay-out and readability level of the IFUs was assessed as previously described [12]. Font size was measured in Cicero as the 'kp' distance. For health instructions in general, font sizes >12 are recommended, interline spacing ≥ 2 and fonts of open letter types [16]. The readability level was expressed as Flesh Kincaid grade level. There are no criteria of readability levels for IFUs but for patient education files, a level $\leq 6^{th}$ grade is recommended [16].

Some RDT products (OptiMAL and TODA) were delivered as boxes containing multiple single-use RDT packages with IFUs supplied in the boxes as well as in the individual packages. In these cases only the IFUs included in the individual packages were considered, as this IFU will most likely be the one available to the traveler.

Statistical Analysis

For *P. falciparum* diagnosis, sensitivity was defined as follows: the number of *P. falciparum* samples with a visible *P. falciparum* specific test line, divided by the total number of *P. falciparum* samples. For the non-*falciparum* species, sensitivity was defined as the number of non-*falciparum* samples with a visible pan-pLDH test line, divided by the total number of non-*falciparum* samples. As one product (OneStep, Table 2) detects *P. vivax* instead of all non-*falciparum* species, sensitivity was defined as all *P. vivax* samples with a visible *P. vivax* test line divided by the total number of *P. vivax* samples. Sensitivity was calculated with 95% confidence intervals (C.I.).

Table 1. Panel of clinical samples used to assess the test characteristics of the RDT products.

		Region of travel			
Species	**Parasite density/μl median (range)**	**Africa**	**Asia**	**Caribbean/South-America**	**No data**
P. falciparum (n = 55)	2,928 (21–1,750,000)	51		3	1
P. vivax (n = 15)	1,068.5 (15–14,228)	6	6	1	2
P. ovale (n = 15)	817.5 (51–5,930)	14			1
P. malariae (n = 5)	382 (26–1,920)	5			1
Negative for malaria (n = 10)	–	8	1		1

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Table 2. Overview of RDT products and their lot numbers.

Product name	Manufacturer	Further referred to as	Target antigens	Lot numbers	CE label	Recommended storage temperature	Number of tests per kit	Price per test (€)	Link to the product*
Carestart™ Malaria HRP2/pLDH (Pf/PAN) COMBO Test	Access Bio, Inc., New Jersey, USA	CareStart	PfHRP2/ pan-pLDH	AIIIR F40IR	Yes	4–30°C	5	7	
Malaria Curative Kit, Immunoquick	BioSynex, Strasbourg France	Immunoquick	Pf†	SMD031411 SMD081611	Yes	4–30°C	3	21	http://www.smihealth.com/wp-content/uploads/2010/03/HKP001E2.pdf
Labstix Malaria Travel Kit	Labstix Diagnostics (Pty) Ltd., Faerie Glen, South-Africa	Labstix	PfHRP2/ pan-pLDH	F1122G1B	No	No temperature displayed	3	6	http://labstix.co.za/products-page/infectious-diseases-other/labstix-malaria-travel-kit/
One Step Malaria (P.F/P.V) Test	Not displayed	OneStep	Pf/Pv†	2010070906	No	4–30°C	1	18	www.std-home-test.com/malaria.html
OptiMAL-IT	Bio-Rad, Marnes-la-Coquette, France	OptiMAL	Pf-pLDH/ pan-pLDH	OAOO35M	Yes	2–30°C	24	2.5	www.tcsbiosciences.co.uk/optimal_it.php
Malaria Combo Test	Sanitoets Closed Corporation, Pretoria, South-Africa	Sanitoets	PfHRP2/pan-pLDH	50034 5000	No‡	No temperature displayed	2	12.5	www.anytestkits.com/malaria-test-kit.htm#Buying from us
SD BIOLINE Malaria Ag P.f/Pan POCT	Standard Diagnostics, Hagal-dong, Korea	SDFK63	PfHRP2/ pan†	90122	Yes	1–40°C	1	4	
TODA MALARIADIAG 4+	TODA PHARMA, Brussels, Belgium	TODA	PfHRP2/ pan-pLDH	K10M4C030111B-1	Yes	2–30°C	1	10	

PfHRP2 = *P. falciparum* Histidine-rich protein-2; pan-pLDH = pan *Plasmodium* lactate dehydrogenase; Pf-pLDH = *P. falciparum*-pLDH.
*Websites last accessed on 07-08-2012.
†Target antigens not specified.
‡On the website a CE label was displayed but not on the delivered products.
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False positive test lines were defined as any visible test line among the *Plasmodium* negative samples, non-*falciparum* samples generating a visible *P. falciparum*-specific test line and in addition for OneStep a visible *P. vivax* test line generated by *P. falciparum*, *P. ovale* or *P. malariae* samples.

Inter-observer agreement for positive and negative readings and line intensity was expressed by kappa values (κ). In line with previous RDT evaluations κ 0.60–0.80 was considered good and κ >0.80 excellent.

Blood Lancets and Transfer Devices

The type of blood lancet (*e.g.* safety lancet with retractable needle, safety seal lancet, simple lancet) and transfer device (pipette, straw, loop, inverted cup) was recorded. Besides, blood lancets were assessed for possibility of reuse. The safety lancets with retractable needle consist of a plastic casing in which a needle is fixed. The system has to be primed and next the needle is launched by pushing on a button. For these systems it was checked that the needle could not be launched inadvertently (*e.g.* during transport in the luggage of the traveler) before the protective cap was removed.

Additional Analysis

To assess the presence of histidine-rich protein-2 (PfHRP2) in one *P. vivax* sample showing a *P. falciparum* test line of strong and weak intensity in four and one out of five PfHRP2-detecting RDTs, a PfHRP2 ELISA (Standard Diagnostics, Hagal-Dong, Korea) was performed.

Results

RDT Selection

During the internet search eight RDT products were encountered and ordered. Three of these products were not included in the final selection: one (Malapack Travel test, http://www.vaccinations.com.au/product.htm) was not marketed anymore, the manufacturer of the second product (EZ-Trust Malaria Rapid Screen Test Kit, CS Innovation Pte Ltd, Singapore) replied not to start up the production for an order less than 10,000 tests. The third product that was delivered (Unitest malaria cassette Pf-Pv, Ciriano global S.L., Zaragoza, Spain) appeared to be an RDT detecting malaria antibodies instead of antigens (Figure 1), whereas the product ordered from the manufacturer's website clearly mentioned an antigen-detecting product.

In addition to the five ordered RDT products, the three tests directly provided by the manufacturers (as explained above) were also included, so that a total of eight RDT products was evaluated (Table 2). Six RDT formats were cassettes, there was one hybrid format (OptiMAL) and one RDT (Immunoquick) consisted of a dipstick.

RDT Performance

The number of samples detected and the number of false positive lines for malaria negative samples for each RDT product are displayed in Table 3. For 6/8 RDT products 100% sensitivity was reached for *P. falciparum* diagnosis, but Labstix and Onestep showed false positive lines in respectively 80% and 70% of malaria negative samples. Three RDT products detected all *P. vivax* samples. *P. ovale* and *P. malariae* detection was in general poor, Labstix was the only one diagnosing all samples but this was at the expense of poor specificity as this product generated visible pan-pLDH lines in 8/10 *Plasmodium* negative samples as well.

Cross-reactions of non-*falciparum* samples with the *P. falciparum* test line occurred for the majority of samples in Labstix (94.3%) and OneStep (80.0%). In addition for OneStep 47 *P. falciparum* samples and 12 P. *ovale*/*P. malariae* samples showed a visible *P. vivax* test line.

Faint Test Lines

For *P. falciparum* diagnosis, the median percentage of correctly identified test lines with faint line intensity per RDT product was 3.6% (range 0.0%–14.0%). For the correctly identified non-*falciparum* samples, faint line intensities occurred at a median frequency of 23.8% (range 10%–58.3%).

Interobserver Agreement

Median κ for positive/negative readings for the *P. falciparum* test line was 0.95 (range 0.50–1.00, OneStep κ = 0.50), for the non-*falciparum* test line this was 0.91 (range 0.66–1.00). For line intensity readings median κ were 0.83 (range 0.70–0.96) and 0.76 (range 0.71–0.85) for the *P. falciparum* and non-*falciparum* lines respectively.

Reading Beyond the Recommended Reading Time

For all but one (SDFK63) products false positive test lines were seen after reading beyond the recommended reading time among 10.0%–90.0% of the malaria negative samples and 9.4%–100% of the non-*falciparum* samples with initially no false positive test lines. Immunoquick, TODA and Labstix were mainly affected with nearly half (>44.4%) of malaria negative samples erroneously diagnosed as malaria and the majority (>88.2%) of non-*falciparum* samples diagnosed as *P. falciparum* malaria upon reading beyond the recommended time.

RDT Packaging, Content and Design

Half of RDT products assessed had inconsistencies in their names displayed on the outer packaging, the device packaging and the IFU. Moreover, 3/8 RDT products did not mention their target antigens and 2/8 RDT products did not display recommended storage temperature (Table 2). In general, the RDT products contained all material needed to perform the test, however to open the buffer vial of Labstix, a scissor was needed but this was not mentioned among the required materials, only in the procedural steps of the IFU. Only 2/8 (25.0%) RDT products contained a correct desiccant – *i.e.* provided with a color-based humidity indicator allowing to control for humidity saturation. Two products did not contain a transfer device, the drop of blood had to be applied directly to the test strip. The pipettes supplied with Labstix and OneStep did not contain a mark for indication of the correct volume of blood.

For 4/7 RDT devices test line labeling consisted of acronyms ('Pf', 'P', 'Pan'), the others used either letters (T) or numbers. Immunoquick contained no labeling at all as it is a dipstick without cassette. Incorrect labeling of the reading window was found for Sanitoets: only the symbols 'C' and 'T' were displayed at either side of the reading window while the strip contains a control line ("C") and two test lines. For Labstix discordances were observed between labeling on the test device (Pf, Pan) and the pictures displayed in the IFU (T2, T1).

Instructions for Use

OneStep did not deliver an IFU with the tests, nor a link to the online version. On the website where the product was ordered an IFU was found. The IFU of OptiMAL consisted only of pictures. None of the IFUs fulfilled requirements for correct font size (>12), median font size was 5.0 (range 4.5–9). Only two products used an interline distance of 2 and six IFUs used an open character.

Figure 1. Received product of Unitest (Ciriano global S.L., Zaragoza, Spain). The delivered Unitest malaria cassette P.f-P.v kit mentions detection of antibodies while the almost identical kit on the website mentions detection of the Pf and Pv antigen. (http://www.clinica.co.za/index.php?page=shop.product_details&flypage=flypage.tpl&product_id=11&category_id=3&option=com_virtuemart&Itemid=90).
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Median Flesh Kincaid grade level was 8.32 (5.86–9.65), only Immunoquick had a Flesh Kincaid grade level $\leq 6^{th}$ grade.

Shortcomings of the IFU with regard to test procedure and interpretation are displayed in Table 4. Several critical procedural steps were missing in more than half of the IFUs. For three RDT products, the correct use of the blood lancet was not clearly described or depicted. Sanitoets depicted another type of pipette in the IFU than was delivered in the kit. Moreover, Sanitoets mentioned to cut the end of the sealed pipette that contained the reagent while in the kit a buffer vial with a screw cap was included. Four IFUs failed to mention that the test should not be read beyond the recommended reading time and one IFU (TODA) did

Table 3. Test characteristics of the different RDT products.

	Number of samples identified (%)					False positive lines		
	P. falciparum		*P. vivax*	*P. ovale*	*P. malariae*	Pv, Po and Pm (n = 35)	malaria negative (n = 10)	
RDT product	PD <1,000/µl (n = 15)	PD >1,000/µl (n = 40)	(n = 15)	(n = 15)	(n = 5)	Pf test line	Pf test line	Pan/Pv test line
CareStart	15 (100)	40 (100)	15 (100)	4 (26.7)	3 (60.0)	4 (11.4)*		
Immunoquick	15 (100)	40 (100)				4 (11.4)		
Labstix	15 (100)	40 (100)	15 (100)	15 (100)	15 (100)	33 (94.3)*	8 (80.0)	8 (80.0)
OneStep	15 (100)	40 (100)	12 (80.0)			28 (80.0)	7 (70.0)	3 (30.0)
OptiMAL	11 (73.3)	39 (97.5)†	13 (86.7)‡	1 (6.7)	3 (60.0)	2 (5.7)	1 (10.0)	1 (10.0)
Sanitoets	13 (86.7)	39 (97.5)†	12 (80.0)§	7 (46.7)	2 (40.0)	2 (5.7)*		
SDFK63	15 (100)	40 (100)	15 (100)	1 (6.7)	3 (60.0)	3 (8.6)*		
TODA	15 (100)	40 (100)	14 (93.3)	3 (20.0)	4 (80.0)	1 (2.9)*		1 (10.0)

PD = parasite density, Pf = *P. falciparum*, Pv = *P. vivax*, Po = *P. ovale*, Pm = *P. malariae*.
*including one P. vivax sample that generated a strong positive result upon testing PfHRP2 ELISA.
†one sample missed with parasite density 2,458/µl.
‡including one invalid result. § including one missed sample with parasite density 3,251/µl.
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Table 4. Presence of important items that need to be addressed in the instructions for use.

Procedure section	Care-Start	Immuno-quick	Labstix	OneStep	OptiMAL	Sani-toets	SDFK63	TODA
Do not use the RDT if the device package is damaged	No	Yes	No	Yes	No	No	Yes	No
Do not use beyond the expiry date	Yes	No	No	Yes	No	No	Yes	Yes
Use the device immediately after opening	No	No	No	No	No	Yes	Yes	No
Place the device on a level surface	No	N.A	No	Yes	N.A.	Yes	No	Yes
Check the desiccant for signs of exposure to humidity	No	No	No	No	No	No	No	No
Disinfect finger with alcohol wipe	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Allow the finger to dry before pricking	No	No	No	Yes	No	Yes	No	Yes
Correct use of blood lancet is clearly described/depicted	No	Yes	Yes	Yes	No	Yes	No	Yes
The volume of blood to be transferred is clearly mentioned	Yes	Yes	Yes	No	Yes	No	Yes	No
Hold the buffer vial vertically	No	No	No	No	No	No	Yes	No
Do not use another buffer than the one provided in the kit	No	No	No	No	No	No	No	No
Use an adequate light source for reading	No	No	No	No	No	No	No	No
Do not read beyond the recommended reading time	No	Yes	Yes	Yes	No	Yes	No	No
Interpretation section								
All possible line combinations for invalid test results	No	No	Yes	Yes	No	Yes	Yes	No
All possible test line combinations for positive test results	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Interpretation of a faint test line as positive	No	No	Yes	No	No	No	No	No
Causes of false negative results, in particular low parasitedensities	No	No	No	No	No	No	No	No
Causes of false positive results, *e.g.* presence of the rheumatoid factor	No	No	No	No	No	No	No	No
A negative test does not rule out malaria	No	No	No	No	No	Yes	No	No
In case of a negative RDT result and persistent suspicion ofmalaria repeat the test after 6–12 h or go to a doctor	No	No*†	No*	No	No	No	No	No
Do not use the test to follow-up treatment	No	No	No	No	No	No	No	No‡
In case of a positive RDT result consult a doctor	No	No*	No*	No	No	Yes	No	No

N.A. = not applicable.
*The user is advised to take the treatment included in the kit.
†Repeating the test after 12 hours is advised, independent of persistence of symptoms.
‡The user is advised to use the 'pan' line for treatment follow-up.
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not display a reading time at all (for this product, information about reading time was withheld from the box that contained the single packages used for self-diagnosis).

OneStep did not contain any pictures of possible test results in the interpretation section. OptiMAL and TODA did not mention a visible control line and single visible *P. falciparum* test line as a possible result. Following their instructions, a *P. falciparum* infection always generates both a visible *P. falciparum* and pan-pLDH test line. In the present study however, OptiMAL and TODA showed a single visible *P. falciparum* test line among one and three *P. falciparum* samples respectively, leading to 49/55 (89.0%) and 52/55 (94.5%) correctly identified *P. falciparum* samples respectively. IFUs that did not display all possible combinations for invalid results generally only depicted a test in which none of the lines were visible. For TODA, an error was observed on the figure showing the invalid results, *i.e.* a cassette was displayed with a visible control line and an absent test line. Except for Labstix that depicted a less visible test lines among the positive results, none of the RDT products mentioned to consider a faint test line as a positive one. Likewise, causes of false negative and false positive results were not mentioned. SDFK63 was the single product mentioning that a negative test does not rule out malaria. Hardly any advices about test policy were made, except for TODA mentioning to use the 'pan' line for treatment follow-up of a *P. falciparum* infection and Labstix and Immunoquick advised to start treatment in case of a positive test or in case of a negative test and persistence of symptoms (Table 4).

Blood Lancets and Transfer Devices

Blood lancets and transfer devices for each RDT product are depicted in Figure 2. Five lancets were so-called safety lancets with a retractable needle. For two of them reuse was possible and for the other three the needle could inadvertently be launched before removal of the protective cap. Two products did not contain a transfer device, the drop of blood had to be applied directly to the test strip. The pipettes supplied with Labstix and OneStep did not contain a mark for indication of the correct volume of blood.

Discussion

The present study assessed the diagnostic accuracy of malaria RDT products available for self-diagnosis as well as the quality of packaging and content, the readability level and lay-out of its IFU. Sensitivity was variable and for two products an unacceptable high number of false positive test lines occurred. All but one product yielded false-positive test lines upon reading beyond the recommended reading time. Major shortcomings in IFU were observed among all RDT products and the IFUs were not user-friendly.

Limitations and Strengths

The present study used a limited and selected number of samples, precluding calculation of predictive values and providing wide confidence intervals for non-falciparum results. Furthermore, RDTs were performed by trained personnel which allowed calculation of RDT sensitivity under perfect conditions but the study design did not include actual performance by the intended end-user. Likewise, ease of use and potential errors of RDT performance as well as interpretation by travelers were not assessed. However, the present study used a collection of samples comprising all four human *Plasmodium* species at different parasite densities providing relevant data on diagnostic accuracy. Besides, the systematic evaluation of RDT packaging and its IFU allowed direct comparison between products and assessment of major shortcomings.

Accuracy for the Diagnosis of Malaria

P. falciparum is the most dangerous species and accurate diagnosis should be guaranteed, even at low parasite densities since the non-immune traveler may already have symptoms at

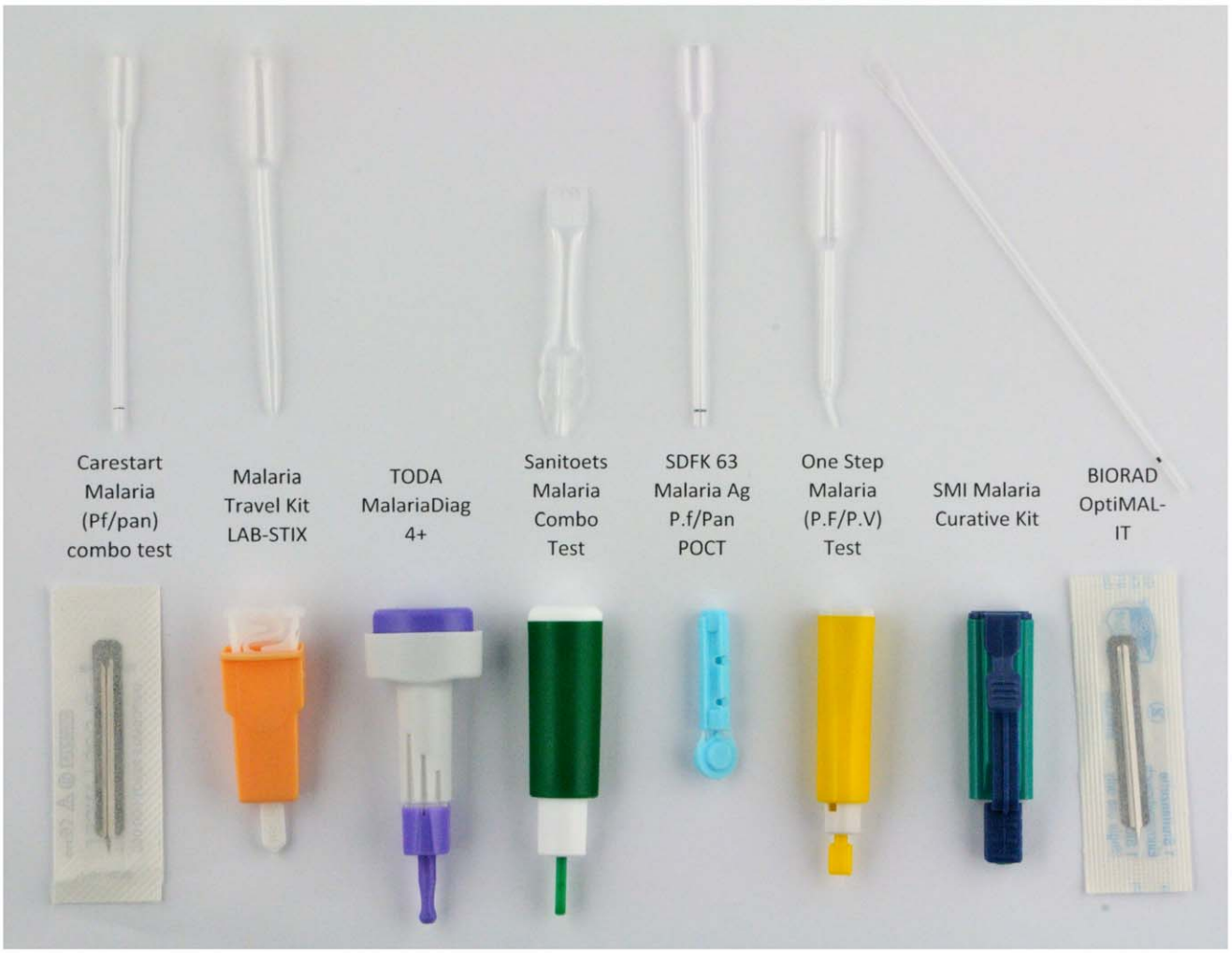

Figure 2. Lancets and transfer devices delivered with the different RDT products. CareStart and OptiMAL included a simple lancet, SDFK63 a safety seal lancet and the other products safety lancets with a retractable needle. The systems of TODA and Immunoquick (SMI) do not require a transfer device (direct contact of the test strip with the drop of blood). Sanitoets contained a calibrated pipette and OptiMAL a straw pipette. The other products included a balloon pipette. The transfer devices of OneStep and Labstix did not display a volume mark.
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parasite densities of 100/µl [17]. Six RDT products had a good sensitivity for *P. falciparum* diagnosis. The use of Labstix and OneStep is however precluded due to the unacceptable high number of false positive test lines. Among the remaining four RDT products, Immunoquick is less suitable as it only detects *P. falciparum* and in most low endemic areas where SBET is considered *P. vivax* is prevalent as well. SDFK63, CareStart and TODA had good sensitivities for both *P. falciparum* and *P. vivax* diagnosis, but detection of *P. ovale* and *P. malariae* was poor, which is a known phenomenon among RDTs [14,18–21]. IFUs should mention these limitations, and advise to repeat a negative test after several hours or to seek reliable health care if symptoms persist.

The high number of false positive test lines for Labstix and OneStep may be due to problems of non-specific binding including buffer composition [22] and a redesign is needed. Moreover, the high number of false positive *P. vivax* test lines for OneStep may be due to wrong citing of the target antigen which presumably detects pan-pLDH rather than P. vivax-pLDH, which has been described previously for other products [23]. The other false positive test lines occurred at random and may be due to non-specific reactions [24].

The *P. vivax* sample showing clearly visible test lines in all PfHRP2-detecting RDT products was probably obtained from a patient with a recent *P. falciparum* infection and PfHRP2 persistence [25] as the presence of PfHRP2 in the blood was confirmed by ELISA.

Faint test line intensity of true positive test lines occurred mainly among the non-*falciparum* species, although for OptiMAL >10% of *P. falciparum* samples also generated a faint *P. falciparum* test line. Faint test lines are of concern as they tend to be regarded as negative [26] and will not be visible under unfavorable light conditions.

It is possible that the ill traveler will check his RDT again after a few hours to make sure it was really negative. After reading beyond the recommended reading time, false positive test lines may occur as demonstrated in the present study, due to the back-flow phenomenon [15]. Therefore, it is important that the IFU clearly mentions that reading test results should be performed within the time specified in the IFU and any test line becoming visible beyond the recommended reading time should be ignored. For OneStep even reading a few minutes too late resulted in some false positive *P. falciparum* test lines observed by the second observer and explaining the low interobserver agreement.

RDT Shortcomings in Labeling

The most serious encountered error in labeling was that on the website of UNITEST malaria antigen detection was clearly mentioned and displayed on the picture, while the product delivered targeted malaria antibodies. Apart from errors in the online ordering system, it is of note that both products are nearly identical except for differences in the text present on the box (Figure 1).

RDT Problems in Design

Some observed errors in design may affect performance under field conditions like errors in labeling of the reading frame and the need of scissors for opening the buffer vial while not mentioned on the package or in the IFU. The lack of a mark on the transfer device to indicate the correct blood volume risks the application of too much or too little blood, leading to poor background clearance obscuring the test lines or to false negative results respectively. The system of direct application of the cassette on the drop of blood looks attractive, but there are no published data about its correctness and ease of use. The most accurate, easy to use and preferred transfer device by health workers in malaria endemic setting was the inverted cup [27] and it can be assumed that this will apply to travelers too.

Instructions for Use: Procedure and Interpretation

The presently found shortcomings in the IFU are of concern as past evaluation showed problems in RDT performance by the ill traveler which improved after revision of the IFU [4]. None of the IFUs was written to the level of the end-user (high readability level, poor lay-out and user unfriendly typography), which is a crucial requirement for products intended for self-diagnosis [28].

Many of the shortcomings mentioned in Table 4 apply to the use of RDTs by any end-user and have been observed in products intended for laboratory use before [12]. Of particular interest to the layman traveler are to check integrity and expiry date of the product as it can be assumed that – despite the long shelf-life of RDTs, storage periods can exceed those indicated by the expiry dates. Moreover, the traveler, and especially the backpacker, will not always be able to adhere to storage conditions (for most RDT products below 30°C) leading to RDT degradation. Further, the curious traveler might open the RDT packaging before intended use, particularly when the IFU is included in a single pouch together with the device, and by doing so he will expose the product to humidity degradation.

The failure to explain/depict the use of the blood lancet is a major shortcoming as one of the major difficulties in RDT performance by travelers observed in previous studies was the finger prick [2,29]. Test interpretation was another frequently observed difficulty [2,3] and therefore all possible test results should preferably be depicted. Of note, information regarding RDT strategic issues (repeat testing, reasons for false negative tests) was poor and when available not always correct *i.e.* using the pan-pLDH line for treatment follow-up, which is debatable, as also gametocytes produce pLDH [30,31].

What can be Done to Improve RDTs for Self-diagnosis?

First of all, an accurate performance needs to be assured. Products like Labstix and OneStep performed insufficiently, they contained no CE mark but were actually delivered to users in the European Union. Next, IFUs should give understandable information about the product performance, including the limitations for the diagnosis of the non-*falciparum* species. Furthermore, the IFU needs to become more user-friendly and the procedure and interpretation sections need to be completed at least with the topics mentioned in Table 4. Also for a traveler, multiple lancets, transfer devices and alcohol wipes are advised. A tag for temperature control (*i.e.* a small device or sticker that changes color when the maximal temperature has been exceeded) may be of additional value. Important, fulfillment of all these requirements does not preclude the need for training and counseling of the end-user of these tests. Although not presently studied, previous reports have demonstrated the needs for training and the benefits of a comprehensive training program [29]. For expatriates and travelers performing RDTs abroad who ask for advice, we currently ask them to send a photograph of the RDT. Future technical developments, such as cell-phone based RDT readers should be assessed for applications [32].

Conclusion

Diagnostic accuracy of currently on the internet available RDTs for self-diagnosis is variable. Based on the present study, 3/8 RDT products are reliable for *P. falciparum* and *P. vivax* diagnosis and one for *P. falciparum* diagnosis only. Instructions on test performance, interpretation and limitations and what to do with test results were

incomplete and unsatisfactory for all RDT products. The presently observed shortcomings need to be urgently adapted before RDTs can indeed be used for self-diagnosis by the traveler and expatriate.

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Author Contributions

Conceived and designed the experiments: JM PG EB AVG JJ. Performed the experiments: JM PG MH. Analyzed the data: JM JJ. Contributed reagents/materials/analysis tools: AVG. Wrote the paper: JM JJ.

Chapter 6. Severe malaria versus invasive bacterial infections

Frequency of severe malaria and invasive bacterial infections among children admitted to a rural hospital in Burkina Faso

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Frequency of severe malaria and invasive bacterial infections among children admitted to a rural hospital in Burkina Faso

Abstract

Background: Although severe malaria is an important cause of mortality among children in Burkina Faso, data on community-acquired invasive bacterial infections (IBI, bacteremia and meningitis) are lacking, as well as data on the involved pathogens and their antibiotic resistance rates.

Methods: The present study was conducted in a rural hospital in Burkina Faso, in a seasonal malaria transmission area. Children (<15 years) presenting with fever and/or signs of severe illness were enrolled upon admission and examined by study nurses. Malaria diagnosis and blood culture were performed for all participants, lumbar puncture when clinically indicated. We assessed the frequency of severe malaria and IBI, and for the latter the causative pathogens and their antibiotic resistance patterns.

Results: From July 2012 to July 2013, a total of 711 patients were included. Severe malaria was diagnosed in 292 (41.1%) children, including 8 (2.7%) with IBI co-infection. IBI was demonstrated in 67 (9.7%) children (bacteremia, n=63; meningitis, n=6), 8 (11.8%) were co-infected with malaria. Non-Typhoid *Salmonella* spp. (NTS) was the predominant isolate from blood culture (32.8%) and mainly isolated after the rainy season, followed by *Salmonella* Typhi (18.8%), *Streptococcus pneumoniae* (18.8%) and *Escherichia coli* (12.5%). High antibiotic resistance rates to first line antibiotics were observed, particularly among Gram-negative pathogens. In addition, decreased ciprofloxacin susceptibility and extended-spectrum beta lactamase (ESBL) production was reported for one NTS isolate each. ESBL production in *E. coli* was observed in 3/8 isolates (37.5%). In-hospital mortality was 8.2% and case-fatality rates for IBI (23.4%) were significantly higher compared to severe malaria (6.8%, $p < 0.001$).

Conclusions: Although severe malaria was the main cause of illness, invasive bacterial infections were not uncommon and had higher case-fatality rates. The high frequency and mortality rates of community acquired IBI require improvement in hygiene, better diagnostic methods and revision of current treatment guidelines.

Introduction

Malaria remains a major cause of mortality in children in most sub-Saharan African countries, despite decreasing incidence rates [1]. Community-acquired invasive bacterial infections (IBI) such as bacteremia and bacterial meningitis also contribute to the under-5 mortality in sub-Saharan Africa [2,3], although exact data are lacking in many areas. Severe malaria and IBI present with similar symptoms [4,5] precluding differential diagnosis on clinical grounds and leading to an overdiagnosis of malaria [6] and ignorance of IBI. In Burkina Faso, a West African country with seasonal malaria transmission, malaria is reported to be responsible for 55% of deaths at district level [7], but data on frequency of IBI, pathogens and susceptibility patterns are hardly available due to a lack of diagnostic microbiology facilities. We conducted a prospective observational study in order to assess proportions of severe malaria and community-acquired IBI among children admitted to a rural hospital and health center in Burkina Faso. In addition, we determined the causative bacteria and their antibiotic resistance patterns and assessed case-fatality rates of both IBI and severe malaria.

Methods

Study site

The survey was conducted at the district hospital Centre Médical avec Antenne chirurgicale (CMA) Saint Camille of Nanoro and the health center Urbain (with inpatient beds), both situated in Nanoro, Burkina Faso. Nanoro is located in a rural area in the Center-West region of the country, approximately 90 km west of the capital Ouagadougou. The health district of Nanoro comprises approximately 153,259 inhabitants of whom 35,159 (22.9%) are children <5 years of age [8]. In Nanoro there is hyperendemic malaria transmission from July – November, corresponding to the rainy season (June – October). Meningitis epidemics may occur in the hot, dry season (February – April), the last dating from 2009, after which a mass vaccination campaign against *Neisseria meningitidis A* was performed [7]. Pneumococcal and meningococcal vaccines were not included in the national extended program of immunization (EPI) at the time of the study period. Vaccination against *Haemophilus influenzae* type b was introduced into EPI in January 2006 and the estimated coverage of immunization was 95.2% in 2011 [8]. In 2010, the overall under-5 mortality rate in Burkina Faso was 129/1000 life-births and 142/1000 life-births in the Center-West region [7]; HIV-prevalence was 1.2% among women 15-49 years and 0.76% among pregnant women [7].

Study population and procedures

All admitted children <15 years of age presenting with axillary temperature ≥38.0°C or clinical signs of severe illness were enrolled, including respiratory distress, prostration, altered consciousness, convulsions, clinical jaundice, hypothermia, signs of shock or severe malnutrition (weight for height score <70% according to national guidelines or kwashiorkor) or with severe anemia (hemoglobin <5 g/dl). Medical history, including prior antibiotic (48h) and antimalarial (2 weeks) treatment, physical examination and outcome of febrile episode (died, referred, discharged) were registered on a standardized form by trained study nurses. Venous blood samples for blood culture, malaria diagnosis, full blood count and blood glucose levels were collected from all participants by trained study staff using aseptic techniques. Lumbar puncture was performed in case of suspicion of cerebral malaria and/or bacterial meningitis, based on the decision of the attending health care worker. Decision for HIV testing or other additional testing was left to the discretion of attending health staff. Treatment decisions were made by the attending health staff according to national guidelines: quinine IV for severe malaria, ampicillin + gentamicin for neonatal infections and ceftriaxone for suspected sepsis or meningitis in older children. Laboratory results were provided instantly to guide treatment decisions.

Laboratory procedures

All laboratory analysis were performed at the Clinical Research Unit of Nanoro (CRUN) which is located on the compound of CMA. Samples were submitted within 15 minutes after collection.

Thick and thin blood films were stained with 3% Giemsa solution (pH 7.2), examined for presence of *Plasmodium* species according to standard procedures [9] and results were expressed as asexual parasites per microliter using the patient's white blood cell (WBC) count. Every slide was read by two experienced microscopists, and in case of discrepant results (positive vs. negative, different *Plasmodium* species, difference in parasite density >Log10 or ratio >2 in case of parasite density ≤400/µl and >400/µl

respectively) by a third experienced microscopist. A selection of slides (5%) was re-read by an expert microscopist whose results were considered conclusive.

The malaria rapid diagnostic test (RDT) recommended by the national malaria control program, SD Bioline Pf (Standard Diagnostics, Hagal-Dong, Korea) detecting *P. falciparum*-specific histidine-rich protein-2 (PfHRP2), was performed on EDTA blood samples according to the manufacturers' instructions.

In all children, 1-3 ml of venous blood was collected into a pediatric blood culture bottle (BD BACTEC Peds Plus^{TM}/F, Becton Dickinson and Company, Sparks, Maryland, USA). A subset of blood culture bottles was weighed before and after blood inoculation to determine the amount of blood collected. All blood culture bottles were incubated in a BACTEC 9050 instrument (Becton Dickinson) for a total of 5 days. If flagged for growth they were Gram stained, subcultured on Eosin-Methylene blue (EMB) agar and 5% Sheep Blood agar (bioMérieux, Marcy-l'Etoile, France) and incubated at 35-37°C for 24 hours in atmospheric conditions and at 5% CO2 respectively. Isolates were identified to the species level by standard biochemical methods and antibiotic susceptibility testing was performed by disk diffusion according to CLSI criteria [10] or in case of assessment of minimal inhibitory concentration (MIC) values by E-test macromethod (bioMérieux) [10]. For *Salmonella* spp., multidrug resistance (MDR) was defined as resistance to the first-line antibiotics ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole (TMP-SMX); reduced fluoroquinolone susceptibility (further referred to as decreased ciprofloxacin susceptibility, DCS) was defined as resistance to nalidixic acid [10] and/or MIC-values for ciprofloxacin >0.06 µg/ml [11]. *Enterobacteriaceae* resistant to cephalosporins were tested for Extended Spectrum Beta-Lactamase (ESBL) production by combined double-disk method (Rosco Diagnostica, Taastrup, Denmark) [10]. For E*nterobacteriaceae*, no breakpoints have been published for azithromycin susceptibility, however EUCAST V 3.1. [11] suggests treatment of infections with *Salmonella* Typhi at MIC-values ≤16 mg/l and recently this value has been proposed as an epidemiological cutoff value for wild-type *Salmonella* spp. [12]. Non-fragile clinically significant organisms were shipped on tryptic soy agar (TSA) at room temperature to the Institute of Tropical Medicine, Antwerp, where identification and antibiotic susceptibility testing were confirmed. *Shigella* spp. and a subset of *Salmonella* spp. isolates was confirmed at the National Reference Laboratory for *Salmonella* and *Shigella* (Institute of Public Health, Brussels) by slide agglutination with commercial monospecific antisera (Sifin, Berlin, Germany), following the Kauffmann-White scheme [13].

Cerebrospinal fluid (CSF) was assessed for its aspect, cell count, Gram stain and culture. The CSF leucocyte count was assessed in a Nageotte counting chamber at x 400 magnification, and if ≥50 WBC/µl were counted a thin film was prepared and stained with methylene blue to count the percentage of neutrophils and lymphocytes. For culture, 1 to 2 drops of CSF were inoculated onto blood agar and chocolate blood agar supplemented with Isovitalex^{TM} (Becton Dickinson) and incubated at 35-37°C at 5% CO_2 for 48 hours. Any bacterial growth observed was further processed by standard microbiological techniques. CSF samples were further assessed by latex agglutination testing for *Haemophilus influenzae b, Streptococcus pneumoniae, Streptococcus* group B, *Neisseria meningitidis* and *Escherichia coli* K1 (Pastorex^{TM} Meningitis , BioRad, Marnes-la-Coquette, France). In addition if *N. meningitidis* was identified serotyping was performed (BioRad or Becton Dickinson).

Full blood counts were assessed using Sysmex XS1000i (Sysmex Corporation, Kobe, Japan).

For determination of glucose levels full blood was collected on NaF tubes and processed by Flexor Junior (Vital Scientific, Netherland). Screening for HIV was performed at the HIV center on the CMA compound according to national guidelines by the rapid diagnostic test Determine^{TM} HIV-1/2 (Alere Medical Co., Ltd, Chiba, Japan) and in case positive the SD Bioline HIV-1/2 3.0 (Standard Diagnostic INC, Kyonggi-do, Korea) was performed.

Case definitions

Fever was defined as axillary temperature ≥37.5°C or reported history of fever in the past 48 hours.

Malaria was defined as the presence of asexual *P. falciparum* parasites confirmed by microscopy.

Severe malaria was defined as microscopically confirmed malaria and fulfillment of at least one of the WHO clinical or laboratory criteria of severe malaria [14] with slight adaptations: respiratory distress was defined as abnormal deep breathing, subcostal retraction or tachypnea according to age [15]; shock

was defined as an abnormal low systolic blood pressure according to age [15], or a combination of temperature gradient (warm trunk and cold extremities) and capillary refill >3 seconds.

Cerebral malaria was defined as severe *falciparum* malaria with coma (Glasgow coma scale <11 or Blantyre coma score <3) or convulsions in the past 24 hours (≥2 or postictal phase ≥30 minutes) and exclusion of bacterial meningitis or hypoglycemia alone (prompt recovery of consciousness after glucose infusion) as cause of symptoms [14].

IBI was defined as bacteremia and/or bacterial meningitis. Bacteremia was defined as the growth of clinical significant organisms from blood culture. Non-pathogenic bacteria or skin flora were considered contaminants, including coagulase-negative *Staphylococci*, *Bacillus* spp. and *Micrococcus* spp.

Bacterial meningitis was defined as i) cerebrospinal fluid (CSF) culture grown with an organism of known clinical significance, or ii) CSF white blood cell count >10/µl with on Gram stain slides the presence of organisms of known clinical significance, or iii) CSF white blood cell count >10/µl and blood culture grown with an organism of known causative role in meningitis or iv) positive latex agglutination. Probable meningitis was defined as a child with clinical signs of meningitis, in whom no lumbar puncture had been performed and a bacteria known to be a causative agent in meningitis was isolated from blood culture.

Data management and analysis

Clinical and laboratory data was double entered in Epi Info version 3.5.3. Statistical analysis was done with Stata 11 (Stata Corp., College Station, TX, USA).

Categorical data were assessed for significance using the chi-square test or Fisher exact test as appropriate. The Wilcoxon rank sum test was used for non-parametric data. Case-fatality rates (CFR) were calculated as proportions of children who died within a specified disease group. Only participants for whom the final hospital outcome was known (discharge or death) were included in the denominator of CFR calculations, referred patients were excluded.

Ethics statement

The study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the national ethics committee of Burkina Faso, the institutional review board of the Institute of Tropical Medicine, Antwerp and the ethics committee of the University Hospital of Antwerp. Written informed consent was given by all parents or guardians of children enrolled.

Results

Patients included

From July 2012 to July 2013, a total of 711 admitted children were enrolled (hospital n=607, health center n=104). Another 177 eligible children were not included for various reasons (not proceeding to study nurse, no blood sampling, refusal to provide informed consent). For age, gender and outcome, there were no significant differences ($p > 0.05$) between included and eligible non-included patients, however, recorded axillary temperature was significantly higher among included patients ($p < 0.001$).

Demographic data, clinical features and laboratory results of the children included are displayed in Table 1. Children were included because of axillary temperature ≥38.0°C (n = 196), signs of severe illness with fever (n = 494) or without fever (n = 21). Lumbar puncture was performed in 19 children. Six children were known as HIV-positive at enrolment and an additional two (out of five tested) were newly diagnosed with HIV infection at inclusion.

Severe Malaria

In total 378 children (53.2%) had microscopically confirmed malaria. Of them, 292 children fulfilled at least one clinical or laboratory sign of severe malaria of whom 48 children (16.4%) had cerebral malaria (Table 2). Severe malaria was more common among children aged 1-5 years than those <1 and >5 years (both $p < 0.001$).

Of note, a positive malaria RDT was observed in 137 children with negative microscopy, among which 106 (77.4%) had at least one clinical or laboratory sign of severe malaria. Of them, 69.8% (74/106) reported previous antimalarial treatment, significantly higher compared to children with microscopically confirmed severe malaria (36.6%, 107/292, $p < 0.001$).

Invasive bacterial infections

Bacteremia was found in 63 (8.9%) children (Table 2), while contaminants were grown in another 27 (3.7%) samples collected. In one blood culture, two pathogens were isolated resulting in a total of 64 clinically significant isolates. Proportions of bacteremia

Table 1. Demographic, clinical and laboratory characteristics upon admission

	All (n = 711)	Hospital (n = 607)	Health center (n = 104)
Female sex, n (%)	318 (44.7)	264 (43.5)	54 (51.9)
Age, median months (IQR)	19 (10-36)	18 (9-34)	28.5 (14-65)
Axillary temperature ≥38.0°C	507 (71.3)	410 (67.5)	97 (93.3)
Pretreatment with antimalarials, n (%)	305 (42.9)	245 (40.4)	60 (57.7)
Pretreatment with antibiotics, n (%)	199 (28.0)	174 (28.7)	25 (24.0)
Clinical features upon admission			
Altered consciousness	33 (4.6)	28 (4.6)	5 (4.8)
Coma, n (%)	23 (3.2)	18 (3.0)	5 (4.8)
Convulsions, n (%)	55 (7.7)	44 (7.2)	11 (10.6)
Prostration, n (%)	304 (42.8)	262 (43.2)	42 (40.4)
Respiratory distress, n (%)	221 (31.1)	172 (28.3)	49 (47.1)
Shock	48 (6.8)	40 (6.6)	8 (7.7)
Jaundice, n (%)	30 (4.2)	30 (4.9)	0
Hemoglobinuria, n (%)	6 (0.8)	5 (0.8)	1 (1.0)
Severe malnutrition*, n (%)	85 (12.7)	80 (13.7)	5 (5.9)
Laboratory features upon admission			
Severe anemia (< 5 g/dl), n (%)	191 (26.9)	186 (30.6)	5 (4.8)
Hypoglycemia (< 2.2 mmol/l), n (%)	36 (5.1)	30 (4.9)	6 (5.8)
Hyperparasitemia (<250,000/µl)	22 (3.1)	16 (2.6)	6 (5.8)

Hb = hemoglobin IQR = interquartile range,

** For 670 children data on severe malnutrition known*

Table 2. Severe malaria and invasive bacterial infections by age group

	All	< 1 m	1 - 11 m	12 - 23 m	24 - 59 m	≥ 60 m
	n = 711	n = 15	n = 195	n = 206	n = 196	n = 99
Severe malaria*, n (%)	292 (42.1)		62 (31.8)	101 (49.0)	101 (51.5)	28 (28.3)
Cerebral malaria, n (%)	48 (6.8)		7 (3.6)	16 (7.8)	22 (11.2)	3 (3.0)
IBI[§], n (%)	67 (9.2)	3 (20.0)	15 (7.7)	20 (9.7)	13 (6.6)	16 (16.2)
Bacteremia, n (%)	63 (8.9)	3 (20.0)	14 (7.2)	18 (8.7)	13 (6.6)	15 (15.2)
Confirmed + probable meningitis, n (%)	6+5 (1.5)	0+1 (6.7)	1+0 (0.5)	3+1 (1.9)	0+1 (0.5)	2+2 (4.0)
Co-infection IBI / malaria, n (%)	8 (1.1)	1 (6.7)	1 (0.5)	3 (1.5)	3 (1.5)	

IBI = invasive bacterial infection, m = months

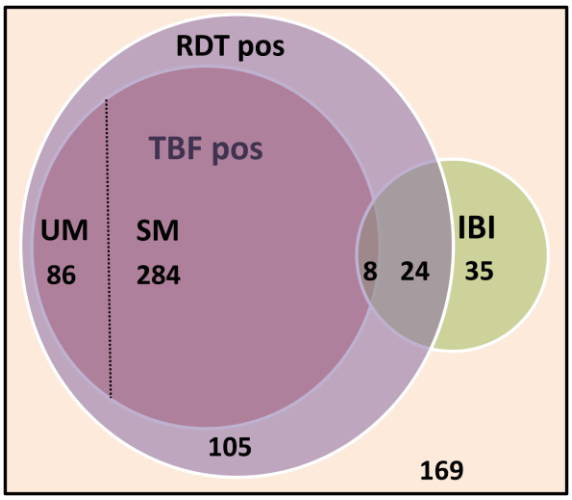
** Including those with IBI co-infection, [§] Including those with malaria co-infection*

were highest among neonates (20.0%) and children ≥5 years of age (15.8%). Pathogens most frequently isolated were non-typhoid *Salmonella* spp. (NTS, 32.8%), *Salmonella* Typhi (18.8%) *Streptococcus pneumoniae* (18.8%) and *Escherichia coli* (12.5%) (Table 3). NTS comprised *Salmonella* Typhimurium (n = 12), *Salmonella* Enteritidis (n = 8) and *Salmonella* Telelkibir (n = 1). *E. coli*, NTS and *Salmonella* Typhi were the most frequently isolated pathogens among children <1 year of age, 1-5 years and ≥5 years of age respectively (Table 3).

The median (range) blood volume per blood culture vial sampled was 0.8 ml (0.3 – 1.2) and 2.1 ml (1.1 – 3.0) for children <2 months and ≥2 months of age respectively. In 19/170 (11.2%) and 46/170 (27.1%) blood culture bottles were under-filled (<0.5 ml) and overfilled (>3.0 ml) respectively.

Bacterial meningitis was confirmed in 6/19 (31.6%) children for whom lumbar puncture was performed; pathogens were *Streptococcus pneumoniae* (n = 3, identified by latex agglutination), *Neisseria meningitidis* W135 (n = 1), *Haemophilus influenzae* (n = 1) and *Klebsiella pneumoniae* (n = 1). There were five additional children with probable meningitis who had *S. pneumoniae* (n = 4) and *N. meningitidis* W135 (n = 1) isolated from blood cultures.

Figure 1. Numbers of children with positive microscopy, RDT and IBI



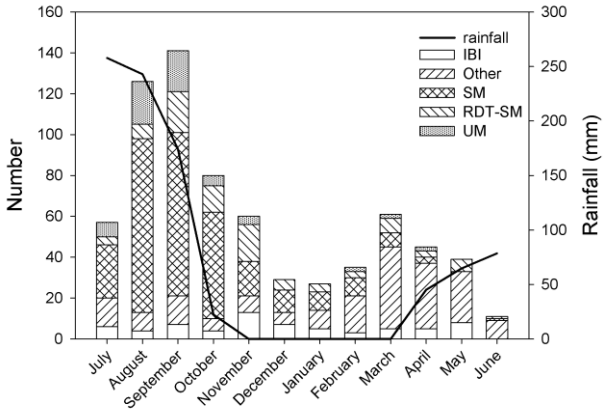
TBF, RDT and IBI results for all children (n = 711), 169 children tested negative on all three items. Areas in Venn-diagram approximately to scale. TBF = thick blood film, RDT = rapid diagnostic test, IBI = invasive bacterial infection, SM = severe malaria, UM = uncomplicated malaria.

Co-infections of severe malaria and invasive bacterial disease

Distribution of positive microscopy, RDT and IBI among children included are displayed in Figure 1. *P. falciparum* parasites were present in 7/63 (11.1%) and 1/6 (16.7%) children with bacteremia and meningitis respectively. Conversely, among the children with severe malaria 8/299 (2.7%) had IBI. No significant difference in parasite densities between children with severe malaria (median 38,319/µl, range 25 – 702,500) and severe malaria / IBI co-infection (79,365.5/µl, range 271 – 338,000) was observed (p = 0.5).

The RDT was positive in 30/63 (47.6%) children with bacteremia and was significantly more frequent among children with NTS (17/21, 81.0%) compared to children with other pathogens isolated (13/42, 31.0%, p < 0.001).

Figure 2. Distribution of severe malaria (SM) and invasive bacterial infection (IBI) by month



IBI = invasive bacterial infection, SM = severe malaria, RDT-SM = signs of severe malaria with negative microscopy and positive RDT, UM = uncomplicated malaria, Other = all other children included not fulfilling criteria of SM or IBI

Proportions of malaria and invasive bacterial infections

The proportion of severe malaria per month ranged from 0.0% - 62.7% among all children included with a peak from August to October (Figure 2), while the proportion of IBI ranged from 0.0% - 24.1% per month with a peak in November – January after the rainy season, mainly due to a high number of NTS infections (62.5%, 15/24 bacteremia cases), and a smaller increase in May.

Table 3. Clinically significant organisms isolated from blood culture and age-specific frequencies

	All	< 1 m	1 - 11 m	12 - 23 m	24 - 59 m	≥ 60 m	Age in months, median (IQR)
All pathogenic bacteria	64	3	14	18	13	16	21 (9 – 54)
Non-typhoid *Salmonella*	21 (32.8)		5 (35.7)	8 (44.4)	6 (46.2)	2 (12.5)	19 (10 – 36)
Salmonella Typhi	12 (18.8)			1 (5.6)	3 (23.1)	8 (50.0)	75.5 (45 – 114)
Streptococcus pneumoniae	12 (18.8)	1 (33.3)	3 (21.4)	5 (27.8)	2 (15.4)	1 (6.3)	12.5 (8.5 – 25)
Escherichia coli	8 (12.5)	1 (33.3)	5 (35.7)		1 (7.7)	1 (6.3)	7.5 (4 – 16.5)
Staphylococcus aureus	3 (4.7)		1 (7.1)			1 (6.3)	
Shigella spp.	2 (3.1)			1 (5.6)	1 (7.7)	1 (6.3)	
Neisseria meningitidis	2 (3.1)			1 (5.6)		1 (6.3)	
Haemophilus influenzae	1 (1.6)					1 (6.3)	
Streptococcus pyogenes	1 (1.6)	1 (33.3)					
Aerococcus viridans	1 (1.6)			1 (5.6)			
Leuconostoc	1 (1.6)					1 (6.3)	

Data displayed are numbers and percentages of clinically significant bacteria isolated.

M = months, IQR = interquartile range

** In one patient 2 pathogens were isolated (E. coli and Leuconostoc)*

Table 4. Antibiotic resistance patterns of *Enterobacteriaceae*

	Non-typhoid *Salmonella*	*Salmonella* Typhi	*Shigella* spp.	*Escherichia coli*	*Klebsiella pneumoniae**
	n = 21	n = 12	n = 2	n = 8	n = 1
Antibiotic	**n (%) resistant isolates**				
Ampicillin	19 (90.5)	0	2	7 (87.5)	1
Chloramphenicol	19 (90.5)	10 (83.3)	1	2 (25.0)	1
TMP-SMX	19 (90.5)	10 (83.3)	2	6 (75.0)	1
MDR	19 (90.5)	0	NA	NA	NA
Nalidixic acid	1 (4.8)	0	0	NA	NA
Ciprofloxacin	NA	NA	0	5 (62.5)	0
DCS	1 (4.8)	0	NA	NA	NA
ESBL confirmed	1 (4.8)	0	0	3 (37.5)	0
Azithromycin	0	0	0	NA	NA
Gentamicin	NA	NA	NA	4 (50.0)	0
Meropenem / Ertapenem	NA	NA	NA	0	0

DCS = decreased ciprofloxacin susceptibility, ESBL = extended-spectrum beta-lactamase, MDR = multidrug resistant, NA = non applicable, TMP-SMX = trimethoprim-sulfamethoxazole

** Isolated from CSF*

Antibiotic resistance patterns of bacterial pathogens

Table 4 displays the antibiotic resistance rates of the *Enterobacteriaceae*: 19/21 NTS isolates were MDR, of them one *Salmonella* Typhimurium was in addition ESBL producing and one *Salmonella* Enteritidis isolate showed DCS (ciprofloxacin MIC-value 0.25 μg/ml, nalidixic acid MIC-value >256 μg/ml). All other NTS isolates were susceptible to ciprofloxacin (MIC-values ≤0.023 μg/ml). *Salmonella* Telelkibir was susceptible to all antibiotics tested. Among the *Salmonella* Typhi isolates, 10/12 (83.3%) were resistant to both chloramphenicol and TMP-SMX but all were susceptible to ampicillin and ciprofloxacin (MIC-values ≤0.008 μg/ml). *Shigella flexneri* was resistant to ampicillin, chloramphenicol and TMP-SMX, *Shigella dysenteriae* was resistant to ampicillin and TMP-SMX. All NTS, *Salmonella* Typhi and *Shigella* isolates were susceptible to azithromycin (MIC-values ≤6.0, ≤2.0 and ≤1.0 μg/ml respectively). Three *E. coli* isolates were confirmed ESBL producers and co-resistant to ciprofloxacin, two of them were also co-resistant to gentamicin. No resistance to the carbapenem antibiotics tested was detected.

All *S. pneumoniae* isolates (n = 11, isolated from blood) were susceptible to ceftriaxone (MIC values ≤0.047 μg/ml in 10/11 and 0.19 μg/ml in the remaining one) and 10/11 were susceptible to penicillin (MIC-values ≤0.032 μg/ml in 9/10 isolates and 0.064 in one). The remaining isolate had MIC-values (0.094 μg/ml) above the CLSI meningitis susceptibility breakpoint (0.06 μg/ml). All *S. pneumoniae* isolates were susceptible to erythromycin but most 9/11 (81.8%) were resistant to TMP-SMX. The *N. meningitidis* isolates were susceptible to penicillin (MIC-values 0.047 and 0.064 μg/ml) and ceftriaxone (MIC-values 0.002 μg/ml for both isolates) as was *H. influenzae* (MIC-value 0.006 μg/ml)

All three *S. aureus* isolates were methicillin susceptible as well as susceptible to clindamycin, gentamicin and TMP-SMX; three and one of them were resistant to tetracycline and erythromycin respectively.

A total of 28.2% (205/728) children reported antibiotic use <48h prior to sampling; most frequently administered antibiotics were TMP-SMX (35.1%) and amoxicillin or ampicillin (45.9%). Bacteria were significantly more frequently isolated among children on antibiotics (13.1%, 26/199) compared to those who reported no prior antibiotic use (7.3%, 37/510, p = 0.015).

Case-fatality rates

For 697 (98.0%) children hospital outcome was known: 58/697 (8.3%) children died, of them 20 (34.5%) and 15 (25.9%) were diagnosed with severe malaria and IBI respectively. CFR were significantly higher among children with IBI (23.4%) versus severe malaria (6.8%, p < 0.001), none of the children with severe malaria/IBI co-infection (n = 8) died. Among children with a positive RDT and negative microscopy with signs of severe malaria 11/110 (10.0%) died, of them six had IBI.

CFR for IBI was highest among children <1 year of age (52.9%, 9/17). Among children with bacteremia, deaths occurred among children infected by *S. pneumoniae* (4/12), *E. coli* (4/8), NTS (3/20), *S. aureus* (2/3) and *N. meningitidis* (1/2). None of the patients with *Salmonella* Typhi bacteremia died. Among children with confirmed and probable meningitis 4/10 (40.0%) died (three *S. pneumoniae*, one *N. meningitidis*), while 8/46 (17.4%) children with cerebral malaria died.

Discussion

The present study showed that, among children ill enough to be admitted to rural health facilities in Burkina Faso, severe malaria was the leading cause of morbidity (one half of the admissions) and mortality (one third of the deaths). However IBI were not uncommon and were associated with a significantly higher mortality rate compared to malaria cases. The relative proportions of severe malaria and IBI in febrile and/or severely ill children differed dramatically according to the seasons as well as the bacterial pathogens isolated, with a peak of severe malaria during the rainy season and a relative increase of IBI (mainly due to NTS) shortly thereafter. High antibiotic resistance rates to first line antibiotics were observed, particularly among Gram-negative pathogens.

This study has several limitations. First, a substantial proportion of children fulfilling inclusion criteria was not included. As age and mortality rate did not differ significantly, non-inclusion probably occurred at random although the lower body temperatures may suggest less severe disease in non-included patients. Second, there are the intrinsic limitations of blood cultures such as the moderate sensitivity (*e.g.* 40 – 80% in the case of typhoid fever [16]), further lowered by antibiotic exposure prior to sampling. In the present study, the first-line antibiotics used prior to sampling

(ampicillin and TMP-SMX) were mostly ineffective to the pathogens retrieved and the percentage of bacteremia was higher among children who were on antibiotics before sampling. Nevertheless, more susceptible and fragile strains, like *S. pneumoniae,* may have been missed. Quality indicators of the current blood culture system were satisfying: the percentage of contaminants (3.7%) was acceptable and lower compared to other studies performed in malaria endemic settings (8 – 14.3%) [2,17–20]. Despite the limited number of isolates, the present study is the first to present surveillance data on antibiotic resistance rates in blood culture isolates in Burkina Faso to the knowledge of the authors. Furthermore, performance of a lumbar puncture was left to the decision of the health care worker, and the actual number of meningitis cases may have been higher as in only nineteen children with clinical signs of cerebral malaria and/or meningitis a lumbar puncture was performed. Health care workers in rural Africa may be reluctant to perform a lumbar puncture, because of little experience, lack of appropriate materials and lack of microbiological facilities. In addition, the case definition of severe malaria might have been too stringent: indeed, some children may have been erroneously excluded from the diagnosis of severe malaria as no parasites were seen by microscopy, whereas the PfHRP2-detecting RDT was positive. This was supported by the significantly higher proportion of antimalarial treatment prior to admission in this group compared to microscopy positive children. On the other hand, some children may have had asymptomatic carriage of *P. falciparum* parasites while being ill due to bacterial or viral infection [21,22]. Furthermore, with the available diagnostic methods we could not establish a final diagnosis for all children included. Finally, the limited number of children for whom HIV testing was performed precluded assessment of associations with IBI and/or severe malaria.

Bacterial co-infections among children with severe malaria were less frequent (2.7%) compared to those reported from other studies (4.6% – 8.3.%) [2,17,23–27] as was *P. falciparum* co-infection among children with bacteremia (11.1% versus 14.7% – 21.6%) [2,19,28]. One of the explanations may be the frequent use of antimalarial treatment before admission. When considering also cases with positive RDT results, the proportion of co-infections was substantially higher (49.2% of children with bacteremia had positive RDT), although still lower than in a recent study from Tanzania (56.9%) [26].

Except for surveillance of meningitis epidemics and a single study on blood culture isolates in a tertiary care urban center [29], there were no previous data about the burden of community acquired bacteremia in Burkina Faso, in particular from rural areas. The high proportion of NTS isolates is consistent with studies performed in other rural malaria endemic settings in sub-Saharan Africa [18,19,24–27,30], although most of them reported NTS infections mainly during (and after) the rainy season [18,24,25], while in the present study NTS was scarce during the rainy season with a peak just after it. Recent reviews found *Salmonella* Typhi to be uncommon in children in rural malaria endemic areas in sub-Saharan Africa [31,32], but this pathogen was relatively frequent in our study compared to others performed in similar settings [2,30,33]. This may be due to specific local environment conditions or to the inclusion of children ≥5 years of age. *Salmonella* Typhi is more common in older children [16,26], but may also occur in children ≤5 [26,34] as shown in the present study. Two recent studies performed in sub-Saharan Africa even found higher incidence rates of *Salmonella* Typhi bacteremia among children 2-5 years compared to children ≥5 [35,36].

Resistance rates among community-acquired pathogens were high, particularly among the *Enterobacteriaceae*. The majority of NTS was MDR as reported previously for other countries in sub-Saharan Africa [19,37–40]. In line with recent findings from DR Congo [40], we observed isolates with DCS and ESBL. Although limited to a single isolate each, this warrants further microbiological surveillance. For *Salmonella* Typhi, high resistance rates to TMP-SMX (recommended as alternative treatment for typhoid fever by the ministry of health of Burkina Faso [41]) and amoxicillin/ampicillin were observed, which were the antibiotics most frequently prescribed at the health center level in our study population. No MDR nor DCS was found for *Salmonella* Typhi, in contrast to reports from other sub-Saharan countries [40,42–45]. This may reflect differences in antibiotic use, but also differences in prevailing isolates. Unlike recent reports from DR Congo [34,40], no resistance was observed for azithromycin, which is an oral reserve antibiotic useful in the treatment of salmonellosis and shigellosis [46].

Of concern was the presence of ESBL with co-resistance to gentamicin and fluoroquinolone antibiotics among *E. coli* (precluding treatment options with commonly available antibiotics), which is in line with ESBL producing pathogens in the environment and community in sub-Saharan Africa [47–49].

All *S. pneumoniae, N. meningitidis* and *H. influenzae* isolates were susceptible to ceftriaxone, which is the recommended treatment for bacterial meningitis in Burkina Faso.

The high number of resistance to first-line antibiotics (ampicillin, TMP-SMX) requires extension of surveillance studies to other populations (*e.g.* adults, outpatients) and regions in the country and evaluation of the current national treatment guidelines.

CFR for IBI were significantly higher compared to severe malaria (23.4% and 6.8% respectively) and existence of IBI as well as appropriate case management should be more emphasized at all levels of health care. CFR for IBI were in line with those found in rural Kenya (28%) [2] and rural DR Congo (19%) [30], but lower than those in urban Malawi (38%) [50] and Tanzania (35%) [28] which may be due to low HIV-positive rate in Burkina Faso [7]; they were higher than those in rural Mozambique (12%) [20] and rural Ghana (9%) studies [19], but this is probably due to the broader inclusion criteria (inclusion of in- and outpatients) in the latter studies. Previous studies reported 4-6 fold higher mortality rates among malaria/bacteremia co-infected children versus children with severe malaria only [17,23]. In the present study none of the children with malaria/bacteremia co-infection died, but the number of co-infections was low. For the RDT positive / IBI co-infections, CFR increased to 7.4% but remained lower than reported in previous studies [17,25].

As malaria may predispose to NTS bacteremia [51,52], reduction of malaria transmission would not only lead to a reduction in morbidity and mortality due to malaria but could also reduce the burden of NTS bacteremia [52]. As main reservoirs for human invasive NTS have to date not been explored extensively [53], containment of risk factors (malaria, HIV, malnutrition [53]) appears the best preventive measure. Introduction of the 13-polyvalent pneumococcal vaccine and vaccination against typhoid fever as well as improved hygiene could further reduce the burden of community-acquired childhood bacteremia in this area.

In conclusion, although severe malaria was the main cause of disease among children admitted to a rural hospital in Burkina Faso, invasive bacterial infections were not uncommon and had higher CFR. The high proportion and case-fatality rate of community acquired bacteremia as well as the high antibiotic resistance rates of the pathogens require improvement in hygiene, better diagnostic methods and emphasize the need of blood culture surveillance and revision of current antibiotic guidelines.

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Chapter 7. PfHRP2 versus Pf-pLDH detection in severe febrile illness

Accuracy of PfHRP2 versus Pf-pLDH detection by malaria rapid diagnostic tests in children presenting with severe febrile illness in a seasonal hyperendemic malaria transmission area in Burkina Faso

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Submitted for publication

Accuracy of *Pf*HRP2 versus *Pf*-pLDH antigen detection by malaria rapid diagnostic tests in children presenting with severe febrile illness in a seasonal hyperendemic malaria transmission area in Burkina Faso

Abstract

Background: In most Sub-Saharan African countries malaria rapid diagnostic tests (RDTs) are now used for the diagnosis of malaria. Most RDTs used detect *Plasmodium falciparum* Histidine rich protein-2 (*Pf*HRP2), though *P. falciparum*-specific parasite lactate dehydrogenase (*Pf*-pLDH)-detecting RDTs may have advantages over *Pf*HRP2-detecting RDTs. Only few data are available on the use of RDTs in severe illness and we therefore compared *Pf*-pLDH to *Pf*HRP2-detection.

Methods: Hospitalized children aged 1 month – 14 years presenting with fever or severe illness were included over one year. Venous blood samples were drawn for malaria diagnosis (microscopy and RDT), culture and complete blood count. Leftovers were stored at -80 °C and used for additional RDT analysis and PCR. An RDT targeting both *Pf*HRP2 and *Pf*-pLDH was performed on all samples for direct comparison of diagnostic accuracy with microscopy as reference method. PCR was performed to explore false-positive RDT results.

Results: In 376/694 (54.2%) children included malaria was microscopically confirmed. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value were 100.0%, 70.9%, 69.4% and 100.0% respectively for *Pf*HRP2-detection and 98.7%, 94.0%, 91.6% and 99.1% respectively for *Pf*-pLDH-detection. Specificity and PPV were significantly lower for *Pf*HRP2-detection ($p < 0.001$). For both detection antigens specificity was lowest for children 1 – 5 years and in the rainy season. PPV for both antigens was highest in the rainy season, because of higher malaria prevalence. False positive *Pf*HRP2 results were associated with prior antimalarial treatment and positive PCR results (98/114 (86.0%) samples tested).

Conclusion: Among children presenting with severe febrile illness in a seasonal hyperendemic malaria transmission area, we observed similar sensitivity but lower specificity and PPV of *Pf*HRP2 compared to *Pf*-pLDH-detection. Further studies should assess the diagnostic accuracy and safety of an appropriate *Pf*-pLDH-detecting RDT in field settings and if satisfying replacement of *Pf*HRP2 by *Pf*-pLDH-detecting RDTs should be considered.

Background

Malaria rapid diagnostic tests (RDTs) are currently rolled out in Sub-Saharan Africa to fulfill the need of parasite based diagnosis *e.g.* the parasitological confirmation of malaria before start of treatment [1]. The operational characteristics of RDTs have been extensively evaluated for uncomplicated malaria [2] and the parasite-based diagnosis strategy has proven to be safe in uncomplicated malaria [3]. In contrast, only few studies addressed the use of RDTs in children presenting with severe illness. Those performed reported low specificity of *Plasmodium falciparum* Histidine-rich protein-2 (*Pf*HRP2)-detecting RDTs [4,5], which is most probably due to *Pf*HRP2 persistence after clearance of infection [6]. An alternative would be an RDT detecting *P. falciparum*-specific parasite lactate dehydrogenase (*Pf*-pLDH), which is more rapidly cleared from the bloodstream, but lower sensitivities compared to *Pf*HRP2 have been reported [4]. However recent evaluations of other *Pf*-pLDH-detecting RDT products have shown better performance, also at low parasite densities [7-9]. The RDT used by the national malaria control program of Burkina Faso detects *Pf*HRP2, which is recommended by the World Health Organization (WHO) [10] and used in most Sub-Saharan African countries. The current diagnostic algorithm in Burkina Faso recommends treatment of malaria in case of a positive test and search for other causes of disease when negative [11], but does not differentiate between severe and non-severe disease. The aim of this study was to compare *Pf*-pLDH to *Pf*HRP2-detecting RDTs in children presenting with severe febrile illness in a seasonal hyperendemic malaria transmission area.

Methods

Study site and population

A one year survey (July 2012 – 2013) to assess proportions and incidence rates of invasive bacterial infections and severe malaria was performed in a rural area in the Center-west region of Burkina Faso. In this region there is seasonal hyperendemic malaria transmission and the estimated under-5 mortality in 2010 was 142/1,000 live births [12]. Details of the study have been published elsewhere [13]. In summary, children (< 15 years) presenting with axillary temperature ≥ 38.0°C and/or clinical signs of severe illness who were admitted to the hospital or health center were enrolled. Signs of severe illness included convulsions, altered consciousness, prostration, respiratory distress, shock, hypothermia and severe malnutrition. For the present study children < 1 month of age were not considered.

Sample collection

In all children enrolled blood culture was performed and EDTA-anticoagulated venous blood samples were drawn for malaria diagnosis (both microscopy and rapid diagnostic test). Laboratory analysis was performed in the clinical research unit of Nanoro (CRUN), located on the compound of the district hospital. Leftovers of EDTA blood samples were stored at -80°C within a maximum of 2 hours after sampling until further analysis. Medical history, including previous antimalarial treatment, and clinical examination were registered on standardized forms by trained study staff.

Laboratory procedures

Thick blood films (TBF) were stained with Giemsa and assessed for the presence of *Plasmodium* parasites according to standard procedures [14]. Parasite density was expressed as asexual parasites per microliter using the patient's white blood cell (WBC) count. TBF was considered negative if no parasites were seen on 100 fields. Every slide was read by two experienced microscopists blinded to each other's results and in case of discrepant results (positive vs. negative, different *Plasmodium* species, difference in parasite density >Log10 or ratio >2 in case of parasite density ≤400/µl and >400/µl respectively) by a third experienced microscopist.

The RDT SD Bioline Malaria Antigen P.f (Standard Diagnostics, Hagal-Dong, Korea), further referred to as SD50 (LOT 082160), is the RDT recommended by the national malaria control program of Burkina Faso and detects the protein *Pf*HRP2. SD50 was performed on EDTA blood samples by trained CRUN laboratory staff before slides were read and within a maximum of 2 hours after sampling.

Blood culture work-up and cerebrospinal fluid analysis was performed according to standard microbiological procedures as described previously [13].

Malaria rapid diagnostic test evaluated

The RDT SD Bioline Malaria Antigen P.f (HRP2/pLDH) (Standard Diagnostics, Hagal-Dong, Korea), further referred to as SD90 (LOT RDT12002), is a three-band test consisting of a control line and two test lines targeting *Pf*HRP2 and *Pf*-pLDH respectively. Good

performance was reported in previous evaluations [15,16].

SD90 was performed on EDTA blood samples according to the manufacturer's instructions except for replacement of the transfer device by a micropipette. From February to July (n = 276), SD90 was performed on fresh samples side-to-side to SD50 by CRUN laboratory staff. For the remaining samples (n = 420) SD90 was performed on stored samples at the end of the study period by the investigator.

In case of absence of the control line the test was considered invalid and repeated. Test line intensities were scored as negative, faint, weak, medium or strong compared to the control line by a single observer who was blinded to the result of microscopy. After reading, photographs were taken.

Monitoring and Quality control

A selection of slides (5%) was sent to the Institute of Tropical Medicine (ITM) and again read by an expert microscopist whose results were considered conclusive. SD50 and SD90 were ordered at ITM Belgium and shipped to Burkina Faso where they were stored in a temperature controlled room. The actual kit in use was stored in the parasitology laboratory. Temperature and humidity during shipment and storage were monitored using loggers (Ebro Electronic GmBH, Ingolstadt, Germany).

For discordant results between either SD50 or SD90 and microscopy, samples were retrieved from -80°C storage and both RDTs were repeated by the investigator blinded to microscopy results. The result of the repeat testing was considered for analysis in case the first result was performed by CRUN staff. If both the first and repeat testing was performed by the investigator the first result was considered. Photographs taken were verified to exclude clerical errors.

In order to compare *Pf*HRP2 results of SD50 and SD90, the RDTs were performed side to side on 10% randomly selected stored samples. For SD50, these 10% of stored samples were also compared to results obtained when prospectively performed on fresh samples.

Additional analysis: PCR

Real-time polymerase chain reaction (PCR) was performed in case of discordant results between microscopy and RDT. DNA was extracted from 200 ml whole blood using QIAamp DNA blood Mini kit (QIAGEN, Venlo, The Netherlands) or from TBF if needed [17]. DNA was amplified by a species-specific 18S rRNA real-time PCR (*P. falciparum/P. vivax* [18]), the *P. ovale/P. malariae* duplex was run simultaneously to confirm microscopically identified non-*falciparum* species.

Data management, definitions and analysis

Data were double-entered in Epi info software (version 3.5.3). Statistical analysis was done with Stata 11 (Stata Corp., College Station, TX, USA). For the purpose of this study, microscopy was considered as the gold standard. Samples with asexual *P. falciparum* parasites seen on TBF (irrespective of parasite density and either as mono-infection or as mixed infection with *P. ovale* or *P. malariae*) were categorized as *P. falciparum* positive. The remaining samples, including samples with pure *P. falciparum* gametocytemia, *P. ovale* or *P. malariae* as well as those with no parasites seen, were categorized as *P. falciparum* negative. For SD90, sensitivity and specificity were calculated for both *Pf*HRP2 and *Pf*-pLDH test lines. A visible test line in case of *P. falciparum* positive samples was considered true positive, no visible test line false negative. For *P. falciparum* negative samples, the absence of a visible test line was labeled as true negative, a visible line was labeled as false positive. Sensitivity, specificity and predictive values were calculated by age group and season and expressed with 95% C.I.. Differences were assessed for statistical significance using the chi-square test, or Fisher exact test when appropriate, in case of independent data (*e.g.* comparison between the seasons) and with the McNemar test or paired proportion test for dependent data (*Pf*HRP2 vs. *Pf*-pLDH).

Ethical issues

The study was approved by the ethical committee of Burkina Faso and the University Hospital of Antwerp and by the institutional review board of ITM. Written informed consent was obtained from the parent or guardian of each child included.

Results

Study population and malaria microscopy results

During the one-year study period 696 children aged 1 month – 14 years were included [13]. For two children (both *P. falciparum* positive with parasite densities of

62/µl and 35194/µl respectively) there was evidence of a sample switch during storage, both samples were excluded from analysis. The final collection consisted of 694 samples.

Demographic data of participants during the different seasons are displayed in Table 1. Seasons were divided in Rainy season (July – October, monthly microscopy positivity rate 67.9% - 87.9%) post-rainy season (November – February, monthly microscopy positivity rate 31.4% - 40.0%) and hot dry season (March – June, monthly microscopy positivity rate 2.9% - 17.2%).

In 376 (54.0%) children, samples were *P. falciparum* positive, five of them had a mixed infection with *Plasmodium malariae* (n = 3) and *Plasmodium ovale* (n = 2). Median *P. falciparum* parasite density was 43,231.5/µl (25 - 702,500). Among the *P. falciparum* negative samples (318/694, 45.8%), two had *P. ovale* infection and 13 had pure *P. falciparum* gametocytemia.

Diagnostic accuracy of PfHRP2 and Pf-pLDH

No invalid results were observed for SD90. *Pf*HRP2 and *Pf*-pLDH positivity rates are displayed in Figure 1.

Among the *P. falciparum*-positive samples, *Pf*HRP2 was positive in all samples while *Pf*-pLDH missed one (parasite density 62/µl, Table 2), resulting in an overall sensitivity of 100% and 99.5% for *Pf*HRP2 and *Pf*-pLDH respectively (p = 1.0). Among the *P. falciparum*-negative samples, there were 139 and 29 false-positive *Pf*HRP2 and *Pf*-pLDH lines respectively, corresponding to specificities of 56.3% and 90.9% respectively (p < 0.001). In 25 (7.9%) *P. falciparum*-negative samples both test lines were visible: they included eight samples with pure gametocytemia. *Pf*HRP2 was positive in an additional 114 samples, including another four samples with pure gametocytemia. False positive *Pf*-pLDH test lines in the absence of *Pf*HRP2 lines occurred in four samples of which two were *P. ovale* infection and one pure gametocytemia.

Overall, positive predictive value (PPV) for *Pf*HRP2-detection (73.0%), was significantly lower compared to *Pf*-pLDH detection (92.8%, p < 0.001) while negative predictive values (NPV) were similar (Table 3).

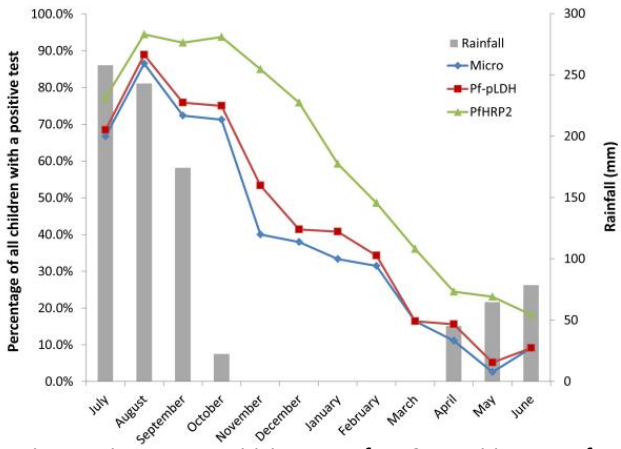
Pf*HRP2 *and Pf-pLDH performance by age group

*Pf*HRP2 specificity was significantly lower among children aged 1 – 5 years (39.6%) compared to children <1 year (70.9%) or > 5 years (68.4%, p < 0.001 for both), for *Pf*-pLDH differences among age groups were not significant (p = 0.053 and p = 0.134 respectively, Table 4). As the slide-positivity rate was highest among children 1-5 years of age, PPV for *Pf*HRP2 in this age group did not differ significantly from the others despite lower specificity (p = 0.428 and p = 0.255).

PfHRP2 and Pf-pLDH seasonal performance

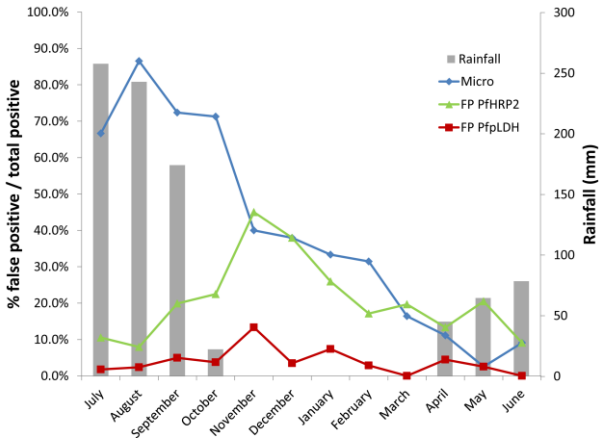
Sensitivity of *Pf*HRP2 and *Pf*-pLDH detection did not differ throughout the year (Table 4). *Pf*HRP2 specificity was significantly higher in the hot dry season (78.9%) compared to the rainy and post-rainy season (35.1% and 46.9% respectively, both p < 0.001). As slide positivity-rate was highest in the rainy season, PPV of *Pf*HRP2 was highest in the rainy season (83.3%), despite lowest specificity, and decreased to 51.9% and

Figure 1. Microscopy, *Pf*HRP2 and Pf-pLDH positivity rate by month



Micro = microscopy positivity rate, PfHRP2 = positive rate of PfHRP2, Pf-pLDH = positivity rate of Pf-pLDH

Figure 2. False positive RDT results per month compared to microscopy positivity rate



Micro = microscopy positivity rate, FP PfHRP2 = % false positive results among total PfHRP2 positive, FP Pf-pLDH =% false positive Pf-pLDH among total Pf-pLDH positive

Table 1. Demographic profile and diagnosis of children included during the different seasons

	All year	Rainy season	Post-rainy season	Hot dry season
	n = 694	n = 398	n = 151	n = 145
Age, median months (IQR)	20 (11 - 37)	21 (12 - 37)	16 (9 - 35)	17 (9 - 39)
Female sex, n (%)	310 (44.7)	176 (44.2)	79 (52.3)	55 (37.9)
Prior antimalarial treatment, n (%)	302 (43.5)	156 (39.2)	87 (57.6)	59 (40.7)
Microscopy *Pf* positive	376 (54.2)	304 (76.4)	55 (36.4)	17 (11.7)
Pf parasite density (/µl), median (range)	42,331 (25 - 702,500)	49,962.5 (62 - 702,500)	18,256 (99 - 259,685)	7,549 (25 - 112,465)
Blood culture positive, n (%)	60 (8.7%)	19 (4.8)	27 (17.9)	14 (9.7)
Confirmed meningitis, n (%)	6 (0.9)	1 (0.3)	2 (1.3)	3 (2.1)
Co-infections	7 (1.0)	4 (1.0)	3 (2.0)	0

IQR = interquartile range, n = number, *Pf* = *P. falciparum*

Table 2. *Pf*HRP2 and Pf-pLDH results according to parasite density

		*Pf*HRP2 pos		*Pf*HRP2 neg	
Microscopy	Number	Pf-pLDH pos	Pf-pLDH neg	Pf-pLDH pos	Pf-pLDH neg
1 - 100	3	2	1		
100 - 1,000	25	25			
1,001 - 10,000	66	66			
10,001 - 100,000	199	199			
> 100,000	83	83			
pure gametocytemia	13	8	4	1	
Microscopy negative	303	17	110	1	175
P. ovale	2			2	
Total	694	400	115	4	175

Neg = negative, pos = positive, *Pf*HRP2 = *P. falciparum* Histidine-rich protein-2, Pf-pLDH = *P. falciparum*-specific parasite lactate dehydrogenase

Table 3. Diagnostic accuracy of *Pf*HRP2- compared to Pf-pLDH-detection

	*Pf*HRP2		Pf-pLDH		*p*-value
RDT pos, n (%)	515	(74.2)	404	(54.2)	
SE	100.0	(94.7 - 100.0)	98.7	(93.5-99.9)	1.0
Sp	70.9	(67.4 - 70.9)	94.0	(90.6 - 94.8)	< 0.001
PPV	69.4	(65.7 - 69.4)	91.6	(86.8 - 92.7)	< 0.001
NPV	100.0	(95.1 - 100.0)	99.1	(95.5 - 100.0)	1.0

N = number, NPV = negative predictive value, *Pf*HRP2 = *P. falciparum* Histidine-rich protein-2, Pf-pLDH = *P. falciparum*-specific parasite lactate dehydrogenase pos = positive, PPV = positive, predictive value SE = sensitivity, Sp = specificity

Table 4. Diagnostic accuracy of *Pf*HRP2 versus Pf-pLDH by age group and by season

	Number	Slide Pf pos (%)	*Pf*HRP2				Pf-pLDH			
			SE (95% C.I.)	Sp (95% C.I.)	PPV (95% C.I.)	NPV (95% C.I.)	SE (95% C.I.)	Sp (95% C.I.)	PPV (95% C.I.)	NPV (95% C.I.)
Age group										
< 1 year	194	39.7	100.0	70.9	69.4	100.0	98.7	94.0	91.6	99.1
			(94.7 - 100.0)	(67.4 - 70.9)	(65.7 - 69.4)	(95.1 - 100.0)	(93.5 - 99.9)	(90.6 - 94.8)	(86.8 - 92.7)	(95.5 - 100.0)
1 – 5 years	401	64.1	100.0	39.6	74.7	100.0	100.0	86.8	93.1	100.0
			(98.3 - 100.0)	(36.6 - 39.6)	(73.5 -74.7)	(92.5 - 100.0)	(98.4 - 100.0)	(84.0 - 86.8)	(91.6 - 93.1)	(96.7 - 100.0)
≥ 5 years	99	42.4	100.0	68.4	70.0	100.0	100.0	94.7	93.3	100.0
			(91.1 - 100.0)	(61.9 - 68.4)	(63.8 - 70.0)	(90.4 - 100.0)	(92.3 - 100.0)	(89.1 - 94.7)	(86.2 - 93.3)	(94.0 - 100.0)
Season										
Rainy	398	76.4	100.0	35.1	83.3	100.0	99.7	85.1	95.6	98.8
			(98.7 - 100.0)	(30.8 - 35.1)	(82.2 - 83.3)	(87.6 - 100.0)	(98.2 - 100.0)	(80.5 - 86.1)	(94.2 - 95.9)	(93.4 - 99.9)
Past-rainy	151	36.4	100.0	46.9	51.9	100.0	100.0	87.5	82.1	100.0
			(92.7 - 100.0)	(42.7 - 46.9)	(48.1 - 51.9)	(91.1 - 100.0)	(93.2 - 100.0)	(83.6 - 87.5)	(76.5 - 82.1)	(95.5 - 100.0)
Hot dry	145	11.7	100.0	78.9	38.6	100.0	100.0	97.7	85.0	100.0
			(78.7 - 100.0)	(76.1 - 78.9)	(30.4 - 38.6)	(96.4 - 100.0)	(81.7 - 100.0)	(95.2 - 97.7)	(69.5 - 85.0)	(97.5 - 100.0)

Pf = *P. falciparum, Pf*HRP2 = *P. falciparum* Histidine-rich protein-2, Pf-pLDH = *P. falciparum*-specific parasite lactate dehydrogenase, pos = positive, SE = sensitivity, Sp = specificity, PPV = positive predictive value, NPV = negative predictive value

38.6% in the other two seasons. Specificity of *Pf*-pLDH detection was in all seasons higher compared to *Pf*HRP2 (Table 4, $p < 0.001$ for each season). Also for *Pf*-pLDH-detection PPV was highest in the rainy season, though differences were smaller: 95.6% in the rainy season compared to 82.1% ($p < 0.001$) and 85.0% ($p = 0.071$) in the post-rainy and hot dry season respectively.

RDT positivity among children with invasive bacterial infections

Microscopy was positive in 8/64 (12.5%) children with invasive bacterial infections (IBI), *Pf*HRP2 and *Pf*-pLDH were positive in 33/64 (51.6%) and 12/64 (18.8%) respectively. As described previously [13] non-typhoid *Salmonella spp.* (NTS) were most frequently isolated from blood culture (21/60, 35.0%). *Pf*HRP2 was positive among 17/21 (81.0%) NTS which was significantly more frequent compared to *Pf*-pLDH (6/21, 28.6%, $p = 0.002$) and microscopy (2/21, 9.5%, $p < 0.001$).

RDT line intensity

For *Pf*-pLDH, 2.4% (9/375) of true positive test lines was of faint intensity, for *Pf*HRP2 this was 0.5% (2/376). For the *P. falciparum* positive samples, the *Pf*HRP2 test line compared to the corresponding *Pf*-pLDH test line for the same sample was of stronger and weaker intensity in 179/376 (47.6%) and 31/376 (8.2%) samples respectively. For seven samples with high parasite density (82,080 – 392,535/µl) *Pf*HRP2 was of weak intensity while *Pf*-pLDH was of strong intensity. Among the false positive test lines (excluding pure gametocytemia), 2/20 (10%) *Pf*-pLDH and 67/126 (53.2%) *Pf*HRP2 lines were of medium or strong intensity.

RDT results of retesting and quality control

During the side to side comparison of *Pf*HRP2 of SD50 and SD90 no discordances between positive and negative results were observed and differences in line intensity were limited to one category.

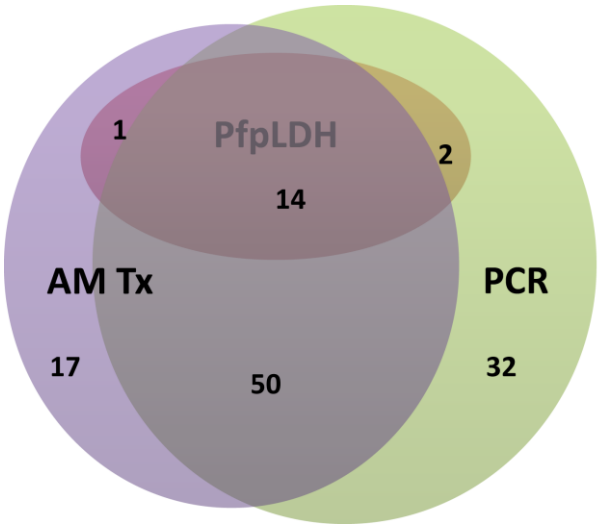
When comparing results of SD50 performed on fresh and stored samples, no differences in positive / negative results nor major differences in line intensities (more than one category) were observed, except for one originally strong result which was faint on repeat testing but for which clerical error could not be excluded.

Further analysis of the false positive samples

In 139/318 *P. falciparum*-negative samples the *Pf*HRP2 line was visible, including 12 samples with pure gametocytemia. For the latter, 10/12 were obtained in children reporting previous antimalarial treatment and 8/12 had a visible *Pf*-pLDH line. Figure 3 summarizes the results of PCR, *Pf*-pLDH and previous antimalarial treatment for the false positive *Pf*HRP2 results (excluding pure gametocytemia) for which PCR was performed (n = 114): *Pf*-pLDH was positive in 16 (14.0%) samples, PCR in 98 (86.0%) and previous antimalarial treatment was reported in 75 (65.8%). For five samples, none of the aforementioned items was positive.

Among both *Pf*HRP2 and *Pf*-pLDH false positive results, report of previous antimalarial treatment was significantly higher (66.2% and 86.2% respectively) compared to microscopy positive samples (33.3%, $p < 0.001$ for both).

Figure 3. Positive PCR and Pf-pLDH results and report of previous antimalarial treatment for false-positive *Pf*HRP2 samples



Only those samples with false positive *Pf*HRP2 for which PCR was performed are displayed (n = 114). AM Tx = previous antimalarial treatment, PfpLDH = positive Pf-pLDH test line, PCR = positive PCR result. For 5 children none of the aforementioned was positive.

Discussion

The present study assessed the diagnostic accuracy of *Pf*HRP2 compared to *Pf*-pLDH antigen detection in

children presenting with severe febrile illness in a rural area with seasonal malaria transmission. Both antigens had excellent sensitivity and similar negative predictive values, but *Pf*HRP2 had a lower specificity resulting in a significantly lower positive predictive value. Specificity was lowest in the rainy season, but due to the high malaria prevalence PPV was highest in the rainy season. The majority of false-positive *Pf*HRP2 lines were PCR positive and/or reported recent antimalarial treatment, part of them were also *Pf*-pLDH positive or had pure gametocytemia.

Limitations

A number of limitations need to be considered. First, although the present study allowed reliable comparison between *Pf*-pLDH and *Pf*HRP2-detection, which was the study objective, the RDT evaluated is not a format that is likely to be used in field settings. However, the simultaneous side-to-side testing of SD90 and SD50 showed similar results, reason why we feel confident to extrapolate the data about *Pf*HRP2 performance to the actual situation in Burkina Faso. Second, RDTs were performed and interpreted by an experienced investigator using a calibrated pipette which may have generated higher sensitivities than would have been obtained under field conditions. Furthermore, 60% of SD90 was performed on stored samples, though samples had been stored for a maximum of one year and had not been thawed prior to testing; in addition, repeat testing on a subset of samples showed no difference in results when performed on fresh or stored samples.

Sensitivity

So far, only two large studies evaluated the diagnostic accuracy of RDTs in children suspected of severe malaria, one in Mozambique and Tanzania [4] and another in Tanzania [5]. The former compared a *Pf*-pLDH with a *Pf*HRP2-detecting RDT: they observed a significant lower sensitivity for the *Pf*-pLDH-detecting RDT (88.0% versus 94.0%), especially at low (< 1000/µl) parasite densities. Of note however, the test used (Optimal-IT) is a multistep RDT with reported lower sensitivities compared to more recent one-step *Pf*-pLDH-detecting RDT products [8,19]. In the present study there was excellent sensitivity of *Pf*-pLDH detection, also at low parasite densities. Only one sample was missed by *Pf*-pLDH detection and this sample had a parasite density of 62/µl, which is below the detection threshold of routine microscopy [20].

Faint test lines, which are prone to be disregarded by health workers [21-23], only occurred in 0.5% and 2.4% of the true positive *Pf*HRP2 and *Pf*-pLDH lines respectively. The co-presence of weak *Pf*HRP2 and strong *Pf*-pLDH lines at high parasite densities (n = 7), may be caused by the prozone effect (false negative/low test lines due to an antigen excess [24]), but was presently not further assessed. Only *Pf*HRP2-detecting RDTs are affected by the prozone effect, not *Pf*-pLDH [24,25]. The degree to which RDTs are affected by prozone is product dependent and in some products *Pf*HRP2 lines may be completely absent [24], leading to false negative results. The weak line intensities at high parasite densities are also of concern, as they may be considered as non-severe disease [23,26].

Specificity

When interpreting specificity several factors should be addressed: first, although in the present study slides were double read by experienced microscopists, very low parasite densities may have been missed. Next, exclusive presence of gametocytes was considered as *P. falciparum* negative (as they do not cause clinical infection) but they produce *Pf*HRP2 and *Pf*-pLDH [27,28] explaining the apparent false positive results. For the purpose of this study we did not consider PCR as the reference method, because it may detect submicroscopic infections (reflecting asymptomatic carriage) which do not explain clinical symptoms [29].

The false positive *Pf*-pLDH lines observed in the present study can in part be explained by a (on-going and partly) treated malaria infection from the previous days as *Pf*-pLDH becomes negative in a median of 2-7 days after start of effective treatment [30,31].This was presently supported by its association with a history of recent antimalarial treatment. In addition, there were three false positive *Pf*-pLDH results for which both *Pf*HRP2 and PCR were negative. Possible explanations may be cross-reaction with pLDH produced by *P. ovale* (n = 2) or other interfering factors [32].

The interpretation of false positive *Pf*HRP2 lines is more complex. The most common cause of false positive *Pf*HRP2 results, especially in high-transmission areas, is *Pf*HRP2 persistence. Other possibilities, though rare, are nonspecific bindings or interference with other immunological or infectious factors such as the

rheumatoid factor, hepatitis C, schistosomiasis, toxoplasmosis, dengue, leishmaniasis, Chagas disease and human African trypanosomiasis [19,33-36].

For the PCR negative samples we can reasonably assume that the false positive *Pf*HRP2 lines were due to past infection, approximately two – six weeks ago. For the PCR positive samples, the subsequent question arises whether the children were actually suffering from malaria at the time of sampling and had negative microscopy because of recently (<2 days) started antimalarial treatment (ongoing infection) [37] or whether it was a recently cleared infection with the child now suffering from another disease. Indeed, microscopy turns negative within 1-2 days after start of artemisinin-based combination therapy (ACT) [38], but the time of PCR to become negative after start of treatment has not yet been studied, although one study reported that upon completion of supervised ACT treatment a third of children were still positive by real time PCR [39]. As the proportion of positive PCR results among false positive *Pf*HRP2 samples was high in our study, we speculate that at least part of the false-positive *Pf*HRP2 lines can be explained by ongoing and partly treated infection, especially in those samples which showed false positive *Pf*-pLDH results as well.

Prior use of antimalarial treatment (either by self-medication or prescription) reflects real-life situation in malaria endemic settings. To know if the child is actually suffering from malaria, an ideal RDT should be able to differentiate ongoing infection from a previously currently cured episode of infection, but *Pf*HRP2 is not capable to do so. *Pf*-pLDH RDTs seem to be more promising in that respect – as they turn negative in 2 – 7 days - but future studies should assess their evolution over time after start of treatment.

Influence of age and season on specificity

The low *Pf*HRP2 specificity in the rainy and post-rainy season compared to the dry season has been observed before [40] and may be explained by malaria infection in the weeks prior to enrolment as malaria transmission is high in these months. In addition children may have had an actual infection and been (partly) treated before enrolment, which also explains the decreased *Pf*-pLDH specificity in the rainy and post-rainy season. For children aged 1 – 5 years, specificity for *Pf*HRP2-detection was extremely low which may be ascribed to their high vulnerability to malaria which was reflected by the high prevalence in this age group. The relation has been observed before [5] while no such association was observed for *Pf*-pLDH-detection, probably due to the more rapid clearance from the blood stream [41-43].

What if treatment is based on RDT results

In children with severe malaria it is crucial that the diagnosis is not missed: a negative RDT should safely exclude malaria. However, overdiagnosis of malaria by diagnostic testing does not only lead to a waste of antimalarial drugs but also increases the risk of ignorance of other possible life threatening diseases like invasive bacterial infections, which is especially true for *Pf*HRP2-detecting RDTs. Even though WHO mentions to look for other causes of severe illness (including IBI) in case of a positive RDT, this strategy is not yet clearly implemented in the diagnostic algorithm of Burkina Faso [11] and may be overlooked in daily reality, especially since tools for diagnosis of other diseases are lacking.

Pf-pLDH versus PfHRP2-detecting RDTs

The present data adds to the debate about *Pf*-pLDH versus *Pf*HRP2-detecting RDTs. The previously reported lower sensitivity and lower heat stability of *Pf*-pLDH-detecting RDTs appeared to be product dependent [19]. Unlike *Pf*HRP2, *Pf*-pLDH-detection is not affected by the prozone effect [24], no gene deletions [15] or antigen polymorphisms [44-47] have been reported and it is rapidly cleared from the blood stream [41,42]. Especially because of its higher specificity, *Pf*-pLDH-detecting RDTs may be more useful in malaria endemic areas. In addition it may be that *Pf*-pLDH-detecting RDTs are more cost-beneficial compared to presumptive diagnosis in high-transmission settings, which was not the case for PfHRP2-detecting RDTs as concluded by Bisoffi et al. [48]. This needs however to be further evaluated.

Future perspectives

To what extent can the present study findings be applied? First, an appropriate *Pf*-pLDH-detecting RDT should be selected and assessed for its diagnostic accuracy and robustness in field studies. Currently only few *Pf*-pLDH-detecting RDT products are available on the international market [32], and only one 3-band RDT targeting *Pf*-pLDH and pan-pLDH and one 2-band RDT targeting pan-pLDH fulfilled WHO criteria of good performance [49]. More emphasis should be on

development and optimization of *Pf*-pLDH-detecting RDTs, and it should be evaluated how fast they become negative during antimalarial treatment. If future field evaluations of *Pf*-pLDH-detecting RDTs are satisfying, the recommendation of the WHO to use *Pf*HRP2-detecting RDTs in *P. falciparum* endemic areas in Sub-Saharan Africa [10] should be reconsidered. In the meantime, diagnostic algorithms should better highlight the possibility of invasive bacterial infections in spite of a positive RDT result.

Conclusion

Among children presenting with severe febrile illness in a region with seasonal hyperendemic malaria transmission, we observed similar sensitivity but lower specificity of *Pf*HRP2 compared to *Pf*-pLDH-detection. Specificity of *Pf*HRP2 was lowest in the rainy season but positive predictive value was highest in this season due to the high malaria prevalence. For each season and age group, the positive predictive value of *Pf*HRP2 was lower compared to *Pf*-pLDH. Part of the apparent false-positive *Pf*HRP2 samples might however be due to parasite clearance after (incomplete or ongoing) treatment with antimalarials at home or at the referring health center. Further studies should assess the diagnostic accuracy and safety of an appropriate *Pf*-pLDH-detecting RDT in field settings and its capacity to distinguish ongoing malaria from recently cleared infections. If satisfying, replacement of *Pf*HRP2-detecting RDTs by *Pf*-pLDH-detecting RDTs should be considered.

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Chapter 8. General discussion and future perspectives

Parts of this discussion have been adapted from

- **Maltha J,** Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. *Clin Microbiol Infect* 2013, 19: 399-407.
- **Maltha J,** Gillet P, Jacobs J. Malaria rapid diagnostic tests in travel medicine. *Clin Microbiol Infect* 2013, 19: 408-415.

General discussion and future perspectives

Malaria RDTs have been developed more than 20 years ago and have undergone many improvements since then and the number of available products has multiplied enormously. Although RDTs are widely available and used on large scale, we observed several shortcomings regarding diagnostic accuracy, appropriateness for end-users and place in the diagnostic algorithm. Here we will discuss our findings with regard to more recently available data and current evolutions in policy. Some of the data presented below have been adapted from two recent reviews we were invited to write about RDTs in travel medicine and RDTs in endemic setting [1,2].

Cross-reactions between the different Plasmodium antigens and antibodies

In Chapter 2 of this thesis, we observed false-positive Pv-pLDH lines (targeting *P. vivax*) in 6/9 RDT products which were tested against a panel of *P. falciparum* samples at high parasite density [3]. A more recent study by Piper et al. found however that, when using antibody combinations most commonly incorporated in RDTs, *P. falciparum* samples with high parasite densities did not generate false positive Pv-pLDH lines [4]. If this is the case, then there may be two other possible explanations. First, non-specific bindings may have occurred, as was shown for some RDT products in Chapter 5 of this thesis which generated positive results in nearly all samples [5]. In our study we did not test the RDTs with *Plasmodium* negative samples to exclude non-specific bindings. However, three out of six RDT products generating false positive Pv-pLDH lines have been evaluated against a panel of *Plasmodium* positive and negative samples. Interestingly, only *P. falciparum* samples generated false positive Pv-pLDH lines, not the *P. ovale*, *P. malariae* or *Plasmodium* negative samples [6–8]. It may be that the *P. falciparum* samples used in chapter 3 contained other interfering factors that have caused false positive results, such as rheumatoid factor, hepatitis C, schistosomiasis, toxoplasmosis, dengue, leishmaniasis, Chagas disease and Human African trypanosomiasis [9–13]. However, as these diseases are rare in the travel population, the expected frequency of these factors among the *P. falciparum* samples tested is much lower than the observed false positive Pv-pLDH lines (8.2 – 29.1%). Therefore it seems plausible that indeed the '*P. vivax* specific' antibodies on the RDT test strip have bound Pf-pLDH. Why this was not found by Piper et al. may be due to the use of different antibody combinations than the ones used in the RDT products we evaluated. In addition Piper et al. used an extra 'washing' step in their analysis which is known to remove weak antibody-antigen bindings. Besides the cross-reactions of *P. falciparum* samples with the Pv-pLDH line, we also observed false positive Pf-pLDH lines in case of *P. vivax* samples [14] and in case of *P. ovale* infection (Chapter 7). Incorrect species diagnosis may lead to incorrect treatment, as discussed in Chapters 2 and 3. Therefore, manufacturers should try to improve the accuracy of their RDT products, which can be done by selection of an ideal combination of detection and capture antibodies [4].

Pfhrp2 gene deletions – local or global problem?

Pfhrp2 gene deletions have first been described in *P. falciparum* laboratory strains in the eighties [15], but recently the absence of this gene has been confirmed in *P. falciparum* field isolates [16]. For how long *P. falciparum* strains with gene deletions have been present in Peru is unknown, although it seems a quite recent phenomenon: a field evaluation of a PfHRP2-detecting RDT in Iquitos in 1998 – 1999 found excellent sensitivity [17]. This study was performed in the same area as where we

observed poor sensitivity of PfHRP2-detecting RDTs due to *pfrhp2* gene deletions. This implies that *P. falciparum* strains lacking *pfhrp2* were only introduced after this period.

Are these gene deletions restricted to the Peruvian Amazon? As the Amazon region where malaria transmission occurs is not only confined to Peru, it would be expected that other South-American countries are affected as well. Indeed, *pfhrp2* gene deletions have been reported from Brazil [18] and the Colombian Amazon [19] and the Centers for Disease Control and prevention (CDC) found *pfhrp2* and *pfhrp3* gene deletions in Bolivia, coastal Colombia and Suriname (Table 1 [20]).

Table 1. *Pfhrp2* and *pfhrp3* gene deletions in different countries assessed by CDC

Country	Samples	*pfhrp2 (%)*	*pfhrp3 (%)*
Bolivia	27	4	68
Colombia	40	7.5	45
Guyana	100	0	0
Peru	94	33	53.8
Suriname	78	14.1	3.84
Honduras	68	0	44

Data retrieved from CDC [20]

More recently, reports from Africa [21,22] and India [23] have appeared. The study in Mali [22] concluded that *pfhrp2* gene deletions only occur in asymptomatic patients, in contrast to our findings [14]. This study was performed 15 years ago, using different techniques and materials. In addition, to conclude that the *pfhrp2* gene was absent, only one PCR was performed: this is of concern as sequence variation at the binding site or low DNA concentration/quality by itself may have caused the negative PCR reaction. By contrast, in other studies and ours we performed two PCRs across the *pfhrp2* gene. Further investigation is warranted to assess if *pfhrp2* gene deletions are indeed present in Mali and how frequently they occur. A recent evaluation of a PfHRP2-detecting RDT performed in Mali found high sensitivities (96% and 95% [24]), making widespread *pfhrp2* gene deletions less plausible. It needs however to be said that in high endemic settings, children are often infected by different *P. falciparum* strains [25]. Therefore, it is possible that many children actually do carry *P. falciparum* strains with *pfhrp2* gene deletions but because of simultaneous infection by *P. falciparum* strains with intact *pfhrp2*, the RDTs remain positive.
In India, in two symptomatic patients with parasite density of 47,136 and 6,952/µl the deletion of *pfhrp2* and *pfhrp3* was confirmed [23] as well as in three patients from Senegal [21].
These findings indicate that *pfhrp2* gene deletions are more widespread than previously thought and the frequency may further increase. Further investigation to identify the extent of the problem are needed and possibly a switch to Pf-pLDH-detecting RDTs if the spread of *pfhrp2* gene deletions increases.

How can end-user performance be improved? [2]
Table 2 lists the most frequent errors committed by end-users in remote settings; they include critical steps related to safety and accuracy of procedure and interpretation. Reading beyond the recommended reading time proves to be a persistent error: at warm ambient temperatures, the excess sample with unbound conjugate flows back by capillary action of the sample/buffer pad and

gets passively deposited on the test band, thereby generating a false positive line, a so-called "Backflow" phenomenon [26].

Many errors by end-users can be explained by shortcomings in packaging, labeling, correctness and readability of instructions [27], as was described in this thesis [5,28], and unsatisfactory comprehension of "universal" ISO15223-based graphical symbols [29]. In addition, shortcomings in other contents of RDT kits may add to poor performance, such as desiccants with absent or difficult to assess humidity indicator [30], tiny alcohol swabs [27], pipette transfer devices with no volume mark and incorrectly packaged lancets. Combined efforts from (repeat) training, clear and simple bench-site job aids (in local language) and supervision visits are effectively and sustainably contributing to correct end-user's performance [31–33].

The currently available RDT products differ from each other on many aspects, *e.g.* lay-out and labeling of RDT device, transfer device to be used and procedure. In countries where two or more tests are deployed or where new different RDT product will replace the old specific training is needed. Similarities between RDT products would facilitate procurement and supply management, as well as training/re-training and supervision. In addition, it is expected that harmonization of RDT products will improve general end-user performance of malaria RDTs in the field. Therefore, the diagnostic work stream of the Roll Back Malaria partnership has developed an action plan for RDT harmonization which is executed by ITM.

Table 2. Errors committed by RDT end-users in malaria endemic settings.

Errors ranked according to chronology of test procedure	Effects/Comments	Countries	References
Not checking expiration date of the device	Confusion may arise from the way of displaying the expiry date [27]	Lao PDR, The Philippines, Uganda, Zambia	[27,31–33]
Not checking the humidity indicator of the desiccant	Humidity weakens the bonds between antibodies and nitrocellulose strip and delays particle resolubilisation [30]	Laos PDR, The Philippines, Zambia	[32,33]
Not using gloves	Gloves protect from blood borne infection transmission	Zambia	[31,32]
Reusing the same gloves for different patients		Uganda, Zambia	[27,31]
Not identifying the cassette with the patients' name or laboratory number	Risk of inversion of results between patients	Zambia	[31,32]
Not cleaning/disinfecting the finger before pricking		Zambia	[27,31–33]
Not allowing the finger to dry after cleaning and before pricking	Antiseptic needs enough action time	Lao PDR, The Philippines, Zambia	[31–33]
Reusing a lancet for a next patient		Zambia	[31]
Desterilizing the lancet before use (by touching the bench or hands)		Zambia	[31]
Pricking the wrong place of the finger (palmar instead of lateral side)	Pricking the palmar side of the finger is more painful than pricking at the side	Zambia	[31]

Not throwing the lancet in a sharps container		Zambia	[31,32]
Dispensing the wrong volume of blood or transferring the blood not completely to the sample well (leaving blood on the wall of the well)	1. Insufficient volume of blood may cause false negative results, 2. Too much blood may increase the risk or the intensity of a prozone effect [34], 3. Too much blood will cause decreased clearance of the strip	Malawi, Sudan, Uganda, Zambia	[27,31,32,35,36]
Distributing blood into the buffer well and/or buffer into the sample well	Sample and buffer well are not always unequivocally labeled [28]	Laos PDR, The Philippines	[33]
Substituting the buffer by another liquid (*e.g.* distilled water)	Use of any other liquid than the buffer provided in the RDT's kit may cause false positive results [37]	Mali, Mozambique	[38]
Dispensing the wrong volume of buffer	1. Insufficient volume of buffer will impede clearance of the strip and/or slow down migration with failure to generate a control line (invalid test results) 2. Too high volume of buffer may cause false positive results due to non-specific bindings	Lao PDR, Sudan, The Philippines, Uganda, Zambia	[27,31–33,36]
Not using a leveled surface to place the cassette	Decreasing the migration time may cause false negative results [39]	DR Congo, Lao PDR, Sudan	[31,33,36]
Not discarding the used materials correctly		Lao PDR, The Philippines, Zambia	[31–33]
Not respecting the correct reading time	1. Reading too early may cause false-negative results, 2. Reading too late may cause false-positive results due to a backflow phenomenon [40]	Lao PDR, Malawi, Sudan, The Philippines, Uganda, Zambia	[27,31–33,35,36]
Disregarding faint or weak test lines	Faint test lines may be difficult to see particularly in unfavorable light conditions (night shifts) and by elderly readers [27,41]	DR Congo, Lao PDR, The Philippines, Zambia	[31–33,42]
Not recognizing invalid test results		DR Congo, Zambia	[32,42]
Interpreting line intensities as indicative for disease severity (and installing treatment accordingly)	Line intensity is not related to severity	Lao PDR, The Philippines, Uganda	[27,33]
Not interpreting correctly a three- band RDT	Difficulties to define the species involved based on the test line results	DR Congo, Sudan	[36,42]

Study subjects were village or community health workers (Cambodia, Lao PDR, Mali, The Philippines and Zambia), staff of peripheral health centers (Malawi, Sudan and Uganda) and hospital laboratory staff (Mozambique and DR Congo). All subjects had been trained in RDT use and performance.
Adapted from Maltha et al. malaria RDTs in endemic setting [2]

Although RDTs were initially mostly performed by laboratory technicians, RDT use is now increasingly expanding to community health workers [43]. Home-based management of malaria (HMM), *i.e.* the diagnosis and treatment of malaria by trained community members, has been recommended by WHO to improve access to effective antimalarial treatment [44]. Promising results

have been reported [45], although appropriate training and continued supervision is needed [31]. Training and supervision may be feasible in research settings, but in remote settings this may be hard to obtain. For example, in DR Congo 50% of health facilities using RDTs had not received any training [42]. Therefore, when implementing RDTs, more emphasis should be put on training and continued supervision of end-users.

Worldwide, approximately 40% of patients with suspected malaria seek treatment in the private sector [43]. RDT regulation and quality control is however lacking in the private sector [46]. This may lead to the use of inaccurate RDT products or inaccurate performance and interpretation due to incomplete or incorrect instructions [28]. Consequently, incorrect diagnosis may be made and the recommended test and treat strategy may not be safe anymore. Therefore, private sectors should be incorporated in the roll-out of malaria RDTs and training of health staff should include those from the private sector.

How can quality of RDTs be guaranteed? [2]

Substandard malaria RDTs are widespread in resource limited settings [47,48] and lot-to-lot variations may affect performance of RDTs [49]. Regulatory approvals from high-income countries are of limited help: for instance, the requirements for the European Union's conformity label (CE Mark) in the case of malaria RDTs are purely administrative [47]. To overcome this vacuum, WHO and partners organized the "Prequalification of Diagnostics Programme": in addition to RDT product dossier assessments, manufacturing sites are inspected for compliance with ISO13485 standards and an active post-marketing surveillance system has been installed [50]. The increasing demand of RDTs (annually need of 1.5 billion RDTs forecasted) may however lead to supply interruptions or decreased quality due to scale-up of the production processes. Further, the so-called WHO/FIND Rounds assess RDTs also for diagnostic accuracy (*P. falciparum* and *P. vivax*) and heat stability [51] and WHO/FIND further offer a lot testing program.

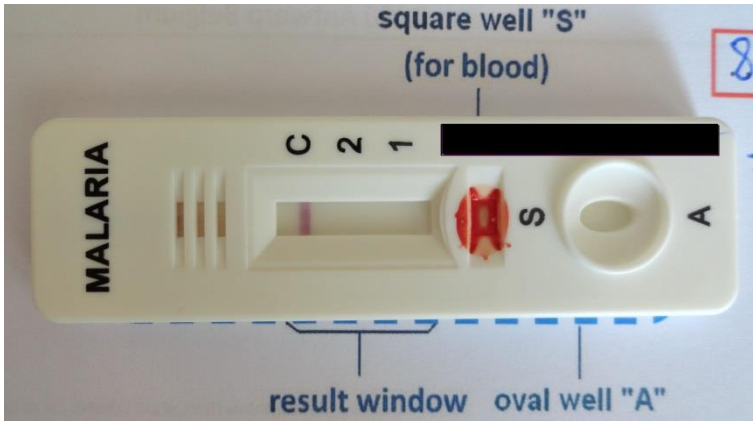
Should RDTs be promoted for self-use by travelers? [1]

As written in Chapter 5, several improvements in RDTs for travelers are necessary with regard to diagnostic accuracy and IFU. The IFU should become more user-friendly, clear information on test accuracy and limitations should be given, and test procedure and interpretation should be completed. In addition we suggested multiple lancets, transfer devices and alcohol wipes as the first attempt may be unsuccessful. However, even with these improvements, we suggest a comprehensive training program and follow-up of results.

In our setting, we offer a training of RDT self-diagnosis to travelers referred by physicians during pre-travel consultations. Travelers are provided with a simple layman-directed step-by-step procedure of the RDT product [52] which includes comments on limitations of RDTs and on what to do with the test results (*e.g.* repeat testing in case of negative result but persistence of symptoms). The training comprises hands-on practice as well as a photo-based quiz about RDT interpretation. Participants are further invited to email photographs of the RDTs they performed on-site for follow-up and advice. To facilitate finger pricking, we replaced the simple "plain lancet" provided in the RDT kit by a "safety lancet" (Sarstedt, Nümbrecht, Germany). Furthermore we provide extra safety lancets, alcohol wipes and transfer devices, as repeat finger pricking and transfer attempts may be expected. From our training sessions, we learned that repeat hands-on practice is essential for acquiring dexterity in finger pricking and transfer of blood; therefore we advise RDT self-testing only to those who have been trained or have practiced RDTs appropriately – it goes without saying that stressful

conditions may decrease the actual performance (Figure 1). RDTs for self-diagnosis may be useful for the traveler when comprehensive instructions and a training program are guaranteed, but further study needs to assess its value under field settings.

Figure 1: Picture of a RDT performed by an expatriate and sent by email

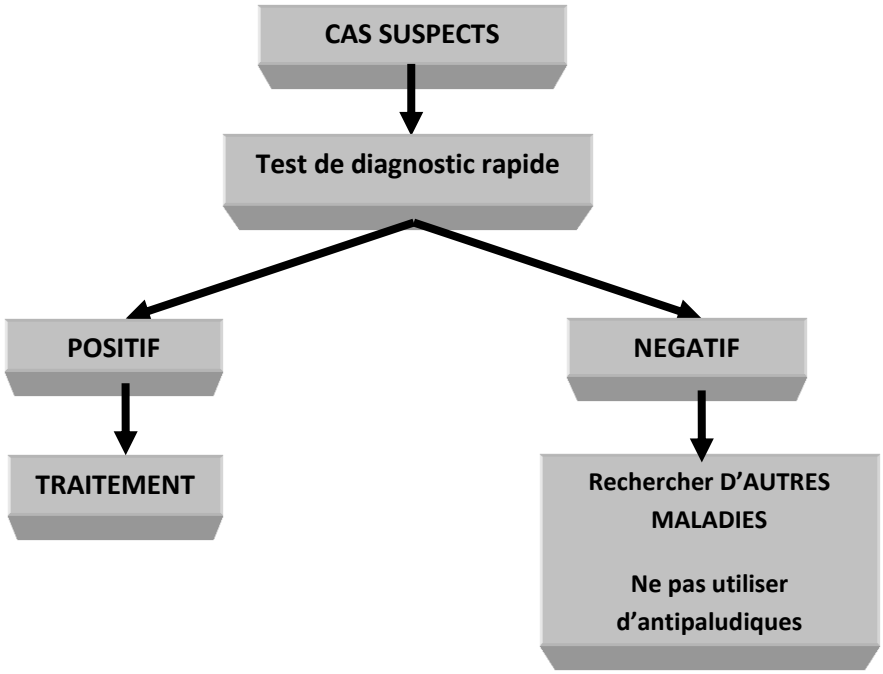


A woman performed the RDT on her 9-year old son in Ivory Coast and sent the picture by email. She had attended the RDT training three months earlier. The cassette is placed on the printed outlines of the layman-directed Standard Operating Procedure [52]. From the picture it is clear that the user had difficulties applying the blood into the sample well (S), as much blood sticks to the plastic casing. Applying a too low volume of blood on the test strip may result in false-negative RDT results. The visible control line (C) only testifies that migration of the colloidal gold complex occurred, but not that the correct sample volume has been applied. Adapted from Maltha et al. Malaria RDTs in travel medicine [1]

Some reflections on the current diagnostic algorithm

The current diagnostic algorithm used in Burkina Faso includes treatment of malaria in case of a positive RDT and withholding antimalarial drugs in RDT negative cases while searching for other causes of disease (Figure 2 [53]). Below, the findings of Chapters 6 and 7 will discussed with respect to the current diagnostic algorithm and current practice in sub-Saharan Africa.

Figure 2 Diagnostic algorithm as displayed in most health facilities in Burkina Faso [53].



Problem 1: "*a positive test is malaria*"

The above quote reflects current practice in many sub-Saharan countries. The recommended RDT type in sub-Saharan Africa detects PfHRP2. As shown in chapter 6 and 7 of the present study and in many others, false positive RDT results are frequent in high-transmission settings due to PfHRP2 persistence after treatment [54–57]. This leads to an overuse of ACTs and it was shown that presumptive diagnosis and treatment of malaria in hyperendemic areas may be more cost-effective than treatment based on PfHRP2-detecting RDTs [58]. Moreover, false positive RDTs lead to ignorance of other diseases that may become fatal. During our study in Burkina Faso, a child had been tested for malaria at a health center and was treated on outpatient basis with intramuscular quinine. On day 2 of treatment the child had become severely ill and was referred to the hospital where the child died upon arrival. It is possible that this child had a bacterial infection with a (false) positive malaria RDT (Chapter6/7), but this could not be confirmed as the child had died before blood sampling could have been done.

Potential measures to prevent overlooking of other diseases include increasing the awareness of other possible life threatening diseases (see below) by training and supervision. In addition, appropriate tools for diagnosis of non-malaria febrile illness should be developed. Another possibility can be the use of malaria Pf-pLDH-detecting RDTs, which are more specific compared to PfHRP2 (see Table 3 for an overview of advantages and disadvantages of Pf-pLDH versus PfHRP2). Due to a higher specificity of Pf-pLDH (because of faster decline and clearance compared to PfHRP2 [54–56,59,60]), less children with bacterial diseases will have false positive RDT results. However, this does not resolve the problem of the 'true' malaria/bacterial co-infections. Of note, Pf-pLDH RDT products consistently showed lower sensitivity compared to Pf-HRP2 RDTs, but recent studies show that well performing Pf-pLDH-detecting RDTs can reach equal sensitivity compared to PfHRP2 (Chapter 7, [54,61]). However, their usefulness in settings where a high number of children are already (partially) treated at the moment of testing needs to be assessed, as discussed in Chapter 7.

Table 3. Advantages and disadvantages of *Pf*HRP2 versus *Pf*-pLDH-detection (Chapter 6)

Item	*Pf*HRP2	*Pf*-pLDH
Sensitivity	Generally high, also at low parasite densities	Lower at low parasite densities, though product-dependent
Specificity	In high endemic settings low due to slow clearance after treatment (> 5 weeks)	Excellent, could be used for treatment follow-up (although debated)
High parasite densities	False negative results may occur (prozone effect)	Not affected by prozone effect
Gene deletions	Reported in South-America and evidence from India and Africa as well	No gene deletions reported
Sequence variation	Highly variable between and within regions, may affect RDT sensitivity	Not reported
Heat stability	Usually up to 45°C	Usually up to 30°C though some products up to 45°C

Problem 2: "*a negative test safely excludes malaria*"

The safety of withholding antimalarial treatment in RDT or microscopy negative children was confirmed in several studies, which reported that withholding antimalarial treatment in test negative children never resulted in severe disease [62–65]. Indeed, most of the RDT products evaluated in

field settings have a very high negative predictive value. In addition, most false negative results occur at very low parasite densities which either may be cleared by the patient himself or may be detected at subsequent testing when the patient consults again with persisting or aggravating symptoms. However, PfHRP2-detecting RDTs may also be negative in case of high parasite densities [66–68], most likely due to the prozone effect [34,69]. And although the prozone effect is relatively rare, missing high parasite densities may become fatal.

Problem 3: adherence to test results

The test and treat strategy has been implemented to restrict malaria treatment only to RDT positive patients. Some studies have reported good adherence to test results [45,62], while in other studies many of the RDT negative patients still received antimalarial treatment [70–74]. In high-transmission settings low adherence to test results may be influenced by initial training, opinion of colleagues, confusing guidelines and patient preference [75]. These factors should be taken into account when implementing RDTs and regular training as well as education of the community may be needed. Moreover, guidance on non-malaria febrile illness may further improve adherence to test results [76].

Problem 4: no alternative diagnosis available

The lack of alternative diagnostic test or clear guidelines may lead to an over-prescription of antibiotics in RDT negative children, as was observed in several studies [62,77,78], probably due to the absence of alternative diagnosis. Over-prescription of antibiotics may further increase antibiotic resistance rates, while the resistance to first line antibiotics is already high in sub-Saharan Africa, as observed in our study and others [79–84]. Clear guidelines on clinical management are needed while RDTs for other causes of febrile illness should be developed and implemented.

Problem 5: diagnostic algorithm for severe malaria

Although children with suspicion of severe malaria should be referred to the hospital urgently, this is not always possible. Long distances, lack of transport facilities, bad road and weather conditions and poor economic circumstances make that some children with 'less' severe disease are diagnosed and treated at health center level. Since RDTs are available, the diagnosis of malaria can now be confirmed at health center level and intravenous quinine can be started. However, as shown in our studies, there is a high number of false positive RDT results and more than half of the children with confirmed bacteremia had a positive RDT. Although training instructions include that a positive thick blood film or RDT does not mean that malaria is the only cause of disease, the possibility of concomitant bacterial infection is not clearly incorporated in the diagnostic algorithm of Burkina Faso and a positive RDT may distract the health care worker from other possible life-threatening diagnosis. A RDT detecting bacteremia would be highly beneficial in these settings to identify those in need of antibiotics, especially since timely diagnosis and treatment for bacterial sepsis is needed to prevent mortality. Existing diagnostic tests for detection of bacteremia (culture, PCR) are time consuming, require special equipment and often fail to detect the low number of bacteria circulating in the blood [85].

An alternative to diagnosis of bacteremia may be provided by the host himself: indeed, metabolomics *i.e.* the study of small molecules released during biochemical processes by the host, can identify specific markers of bacteremia which can be used as basis for an antigen detection test.

Recently we performed a pilot study on the metabolomics-based approach of detecting bacteria in blood. The first results are promising, showing a distinct metabolomics profile in children with bacteremia compared to severe malaria [86]. However, as development and implementation of a RDT may take up to ten years, some other solutions should be sought in the meantime. First, the diagnostic algorithm should emphasize the possibility of a positive RDT with concomitant bacteremia. Second, blood culture surveillance studies, as performed in Chapter 6, should continue and extend to other sites as well, to assess the burden of bacteremia, to identify the most commonly isolated pathogens and to evaluate their antibiotic resistance pattern. This can help to improve clinical guidelines on when to use the appropriate antibiotics.

In conclusion, we provided information with regard to diagnostic accuracy, end-user performance and diagnostic practice, which when addressed by the respective manufacturers, health regulatory authorities or health care workers, can improve malaria diagnosis in children.

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Summary

Malaria is an infectious disease caused by the *Plasmodium* parasite which replicates in red blood cells. Almost half of the world population is at risk of acquiring malaria and 660,000 persons died from malaria in 2010, most of whom were children under the age of five. The World Health Organization (WHO) recommends to confirm the diagnosis of malaria before start of treatment. This confirmation can be done by microscopy, which is often unavailable or of low quality in endemic settings, or by malaria rapid diagnostic tests (RDTs). RDTs are hand-held cassettes that detect malaria by an antigen-antibody reaction. They are easy to use and provide results within 15-20 minutes. Different formats detecting different antigens exist: *Plasmodium falciparum* Histidine-rich protein-2 (PfHRP2), parasite lactate dehydrogenase (pLDH) which can be specific for *P. falciparum* (Pf-pLDH), *Plasmodium vivax* (Pv-pLDH) or common to all *Plasmodium* species (pan-pLDH) and aldolase (all *Plasmodium* species).

In 2010, 88 million RDTs were used worldwide and more than 200 different products from 60 manufacturers were available. Although worldwide used, we hypothesized several limitations and shortcomings regarding i) diagnostic accuracy, ii) appropriateness for end-users and iii) place in diagnostic algorithms.

In **Chapter 2** we showed that in 6/9 RDT products evaluated, *Plasmodium falciparum* samples at high parasite density generated false positive Pv-pLDH lines in 9 – 28% of samples tested, thereby leading to incorrect species diagnosis. In **Chapters 3 and 6** we observed as well false positive *P. falciparum* results in case of *P. vivax* or *P. ovale* samples respectively.

In Peru RDTs could be highly beneficial, especially in remote areas of the Peruvian Amazon where it may take several days before a health center can be reached; however no RDT has been incorporated in the national malaria control program yet. In **Chapter 3** we evaluated a panel of RDTs in the Peruvian amazon and found that PfHRP2-detecting RDTs had low diagnostic sensitivity due to *pfhrp2* gene deletion, which encodes the protein PfHRP2. We showed that Pf-pLDH-detecting RDTs are accurate and hence promising for this region; the Ministry of Health is actually in the decision process upon which RDT product to use.

Clear and complete instructions are crucial to correct performance and interpretation of RDTs by end-users. We assessed a large panel of RDT products for the quality and completeness of their instructions, packaging and labeling (**Chapter 4**). Shortcomings were observed in all RDT products, including some with CE label. Some of the major shortcomings included ambiguous labeling of the reading window, incomplete information about test interpretation and critical differences between depicted device in the instructions and the device present in the RDT kit. Most of these shortcomings can be corrected at low cost and investment and will help to improve end-user's performance.

Nowadays RDTs for self-use by travelers are available through the internet. There is increasing interest in the self-use of RDTs, but quality of these products is unknown. We found that only half of the RDT products advertised for self-testing and purchased through internet could reliably diagnose *P. falciparum* and *P. vivax*. Two products showed positive test lines in nearly all samples, including

the *Plasmodium* negative ones, thereby prohibiting their use. (**Chapter 5**). All products had major shortcomings in the instructions for use with regard to description of test procedure and interpretation, poor readability and lay-out and user-unfriendly typography. Importantly, strategic issues ('what to do if your test is positive or negative') were incomplete or not mentioned at all. Before RDTs can be recommended for self-use by travelers, major improvements need to be done and we only recommend RDTs for travelers when comprehensive instructions and a training program are guaranteed.

Malaria is often over-diagnosed in endemic settings. Severe malaria may present with similar symptoms as invasive bacterial infections, such as bacteremia or meningitis. Co-infections of malaria and bacterial infections may occur as well. In order to improve treatment guidelines in children presenting with severe febrile illness in malaria endemic areas, it is important to know to which extent malaria and invasive bacterial infections occur and what the antibiotic resistance rates are. In a group of severely ill children in a seasonal hyperendemic malaria transmission area in Burkina Faso, we observed severe malaria in 50% of the admissions and invasive bacterial infections in 9%. However, case-fatality rates of invasive bacterial infections were four-fold higher compared to severe malaria. Furthermore, many of the bacteria isolated were resistant against first line antibiotics commonly used in Burkina Faso (**Chapter 6)**.

As malaria rapid diagnostic tests are used in children presenting with severe febrile illness, and as they are the only diagnostic tool available in health centers, we assessed the diagnostic accuracy and predictive value of PfHRP2 versus Pf-pLDH detection in the children studied in Chapter 6. We observed similar (high) sensitivities, but the specificity of PfHRP2-detection was significantly lower compared to Pf-pLDH detection, which was reflected by a lower positive predictive value of PfHRP2 detection for all ages and seasons (**Chapter 7**). Positive PfHRP2 results do not distinguish between recent (<1 week) or ongoing *P. falciparum* infections versus past en cured *P. falciparum* infections more than one week ago. Pf-pLDH probably will be better suited for this purpose as it is rapidly cleared from the bloodstream and should be further studied.

In conclusion, we provided information with regard to diagnostic accuracy, end-user performance and diagnostic practice of malaria RDTs, which when addressed by the respective manufacturers, health regulatory authorities and health care professionals can improve malaria diagnosis in children.

Samenvatting

Malaria is een infectieziekte die veroorzaakt wordt door de *Plasmodium* parasiet die zich in de rode bloedcellen vermenigvuldigt. Bijna de helft van de wereldbevolking loopt risico om malaria te krijgen en in 2010 stierven er 660.000 personen aan malaria, hoofdzakelijk kinderen onder de vijf jaar. De Wereld Gezondheidsorganisatie (WHO) adviseert om de diagnose van malaria te bevestigen alvorens te starten met de behandeling. Deze bevestiging kan gebeuren door middel van microscopie, welke in endemische gebieden zelden beschikbaar is of van lage kwaliteit, of door middel van malaria sneltesten (Rapid Diagnostic Tests, RDTs). RDTs zijn kleine cassettes die malaria detecteren door antigen-antilichaam reactie. Ze zijn gemakkelijk in gebruik en geven een resultaat binnen 15-20 minuten. Er bestaan verschillende formaten die verschillende antigenen detecteren: *Plasmodium falciparum* Histidine-rich protein-2 (PfHRP2), parasiet lactaat dehydrogenase (pLDH) dat specifiek kan zijn voor *P. falciparum* (Pf-pLDH) en voor *Plasmodium vivax* (Pv-pLDH) ofwel gemeenschappelijk kan zijn aan alle *Plasmodium* species (pan-pLDH); daarnaast is er nog aldolase, welke voorkomt bij alle *Plasmodium* species.

In 2010 werden wereldwijd 88 miljoen RDTs gebruikt en er waren meer dan 200 verschillende producten van 60 fabrikanten beschikbaar op de markt. Hoewel RDTs wereldwijd gebruikt worden, vermoedden we een aantal beperkingen en tekortkomingen betreffende i) de diagnostische nauwkeurigheid, ii) de geschiktheid voor eindgebruikers en iii) de positionering van RDTs in diagnostische algoritmes.

In **Hoofdstuk 2** tonen we aan dat *Plasmodium falciparum* stalen met een hoge parasietdensiteit vals positieve Pv-pLDH testlijnen veroorzaakten in 9-28% van de geteste stalen, hetgeen leidde tot een incorrecte diagnose van de *Plasmodium* species, en dit voor 6 van de 9 geëvalueerde RDT producten. In **Hoofdstuk 3 en 6** observeerden we ook vals positieve P. falciparum resultaten voor respectievelijk *P. vivax* en *P. ovale* stalen.

In Peru zouden RDTs van groot nut kunnen zijn, voornamelijk in de afgelegen gebieden van het Peruviaanse Amazonegebied waar het verschillende dagen kan duren voordat een gezondheidscentrum kan bereikt worden; toch zijn de RDTs er nog niet opgenomen in het nationale malaria controle programma. In **Hoofdstuk 3** evalueerden we een panel van RDTs in het Peruviaanse Amazonegebied en we stelden vast dat de RDTs die PfHRP2 detecteren een lage diagnostische gevoeligheid hadden omwille van een *pfhrp2* gen-deletie, het gen dat codeert voor het eiwit PfHRP2. We toonden aan dat RDTs die Pf-pLDH detecteren wel accuraat zijn en dus veelbelovend zijn voor dit gebied; het ministerie van volksgezondheid is momenteel in de beslissingsfase over welke RDT producten zullen gebruikt worden.

Duidelijke en volledige gebruiksinstructies zijn cruciaal voor een correcte uitvoering en interpretatie van RDTs door de eindgebruikers. We hebben een groot panel van RDT producten geanalyseerd voor de kwaliteit en de volledigheid van de gebruiksinstructies in de bijsluiter, hun verpakking en hun etikettering (**Hoofdstuk 4**). We stelden tekortkomingen vast voor alle RDT producten, waaronder ook een aantal met CE label. De meest belangrijke tekortkomingen waren onder meer de dubbelzinnige etikettering van het leesvenster, de onvolledige informatie over de interpretatie van de test en de belangrijke verschillen tussen de weergave van de test in de instructies en de werkelijke presentatie van de test in de RDT kit. In de meeste gevallen kunnen deze tekortkomingen

gecorrigeerd worden aan een lage kost en investering , waardoor de uitvoering van de test door de eindgebruiker gevoelig kan verbeteren.

Tegenwoordig zijn er steeds meer zelftesten voor reizigers beschikbaar op het internet. Er is een verhoogde interesse voor deze zelftesten, maar de kwaliteit van deze producten is nog ongekend. We hebben vastgesteld dat slechts de helft van de RDT producten die op het internet geadverteerd en verkocht werden als malaria zelftesten, een betrouwbare diagnose van *P. falciparum* en *P. vivax* konden geven. Twee producten gaven positieve testlijnen voor bijna alle stalen, ook voor de *Plasmodium* negatieve, waardoor ze niet gebruikt kunnen worden (**Hoofdstuk 5**). Alle producten hadden belangrijke tekortkomingen in de beschrijving van de test procedure en de test interpretatie, de leesbaarheid, de lay-out en de typografie van de gebruiksinstructies. Belangrijker was dat strategische instructies ("Wat te doen als je test resultaat positief of negatief is") onvolledig waren of zelfs helemaal niet genoemd werden. Voordat RDTs aanbevolen kunnen worden als zelftesten voor reizigers moeten er belangrijke aanpassingen gebeuren en ze kunnen enkel aanbevolen worden indien de gebruiksinstructies volledig zijn en indien een trainingsprogramma voor de reizigers kan gegarandeerd worden.

Malaria wordt vaak over-gediagnostiseerd in endemische gebieden. Ernstige malaria kan gelijkaardige symptomen geven dan een invasieve bacteriële infectie, zoals bacteriëmie of meningitis. Co-infecties van malaria en bacteriële infecties kunnen ook voorkomen. Om de richtlijnen voor de behandeling van kinderen met ernstige koorts in endemische gebieden te kunnen verbeteren, is het belangrijk om te weten in welke verhouding malaria en invasieve bacteriële infecties voorkomen en hoe groot de antibiotica resistentie is. Bij een groep van ernstig zieke kinderen in een hyper-endemisch malaria-gebied met seizoensgebonden transmissie in Burkina Faso stelden we bij 50% van de gevallen malaria vast tegenover bij 9% een invasieve bacteriële infectie. De sterfte bij invasieve bacteriële infecties was echter vier keer hoger dan die voor malaria. Bovendien waren vele van de geïsoleerde bacteriën resistent tegen de eerstelijns antibiotica die algemeen gebruikt worden in Burkina Faso (**Hoofdstuk 6**).

Malaria RDTs worden gebruikt voor kinderen met ernstige koorts, en zijn de enige diagnostische methode aanwezig zijn in gezondheidscentra. Daarom evalueerden we de diagnostische nauwkeurigheid en predictieve waarde van PfHRP2 versus Pf-pLDH detectie in dezelfde groep kinderen die bestudeerd werden in Hoofdstuk 6. We observeerden vergelijkbare (hoge) gevoeligheden, maar de specificiteit van de PfHRP2-detectie was significant lager in vergelijking met de Pf-pLDH detectie, wat weerspiegeld werd in een lagere positieve predictieve waarde van PfHRP2 detectie voor alle leeftijden en seizoenen (**Hoofdstuk 7**). Met een positief PfHRP2 resultaat kan geen onderscheid gemaakt worden tussen een actieve of een recent opgelopen infectie (< 1 week) versus een afgelopen en behandelde *P. falciparum* infectie van meer dan 1 week geleden. Pf-pLDH waarschijnlijk meer geschikt om dit onderscheid te kunnen maken, omdat dit eiwit sneller wordt verwijderd uit de bloedbaan. Deze hypothese moet verder onderzocht worden.

Ter conclusie, we hebben informatie verkregen betreffende de diagnostische nauwkeurigheid, de kwaliteit van de uitvoering door de eindgebruikers en op welke manier de RDTs in de praktijk worden gebruikt. Wanneer deze informatie wordt aangeboden aan de respectievelijke fabrikanten, de regelgevende instanties omtrent gezondheid en de gezondheidsmedewerkers, kan dit de malaria diagnosis voor kinderen verbeteren.

Curriculum Vitae

Jessica Maltha (°Holwerd, The Netherlands, 1986) graduated cum laude from the RSG Magister Alvinus in Sneek and started studies of medicine in 2004 at Maastricht University, The Netherlands. During her studies she completed the Honours program "International Health Care", did internships in India and South-Africa and participated to an extracurricular project at the Universidade Catolica de Moçambique, Beira, Mozambique (2008). Becoming interested in tropical medicine, Jessica started in 2009 research at the Institute of Tropical Medicine in Antwerp, which after graduating in 2011 became the start of her PhD research about malaria rapid diagnostic tests. For her research elective during the final year of medical studies (2010), Jessica stayed in Iquitos, Peru where she set up and coordinated a study about diagnostic accuracy of malaria rapid diagnostic test in the Peruvian Amazon. For this project Jessica got awarded with the Student Prize for excellent research of Maastricht University and the Professor Chris Gips student prize 2012 for scientifically most talented medical student in The Netherlands.
In 2011 Jessica obtained a grant from the 'Steunfonds Marguerite-Marie Delacroix' which allowed her to continue her research on malaria diagnosis in children and to complete her PhD training at KU Leuven. As part of this PhD she set up field studies in Nanoro, Burkina Faso (2012-2013), supported by a travel grant from the Junior Mobility program of KULeuven, the Fonds voor Wetenschappelijk Onderzoek (FWO) Flanders and a grant for McKinsey&Company.

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Abstracts and oral communications at scientific meetings

- **J. Maltha**, I. Guiraud, B. Kaboré et al. Invasieve bacteriële infecties versus malaria bij kinderen in Nanoro, Burkina Faso : diagnose bij opname, diagnostisch algoritme en voorspellende waarde van klinische en laboratorium gegevens. Jaarlijks congres van de Nederlandse Vereniging voor Kindergeneeskunde, Veldhoven, The Netherlands. 6-8/11/2013. *Accepted for oral presentation*
- **J. Maltha**, I. Guiraud, B. Kaboré et al. Severe malaria and invasive bacterial infections in children. Congress of the European Society of Pediatric Infectious Diseases (ESPID), 2013, Milan, Italy. *Oral presentation*
- **J. Maltha**, I. Guiraud, B. Kaboré et al. Severe malaria and invasive bacterial infections in children. Voorjaarscongres 2013, Section of tropical pediatrics of the Dutch society of pediatrics. *Oral presentation*
- **J. Maltha**, D. Gamboa, J. Bendezu et al. Rapid diagnostic tests for malaria diagnosis in the Peruvian Amazon: impact of pfhrp2 gene deletions and cross-reactions. International Congress of Tropical Medicine and Malaria 2012, Rio de Janeiro, Brazil. *Poster presentation*
- **J. Maltha**, J. Jacobs. The diagnosis of imported malaria in children. Voorjaarscongres 2011, Section of tropical pediatrics of the Dutch society of pediatrics. *Oral presentation*
- **J. Maltha**, P. Gillet and J. Jacobs. Malaria rapid diagnostic tests: *P. falciparum* infections with high parasite densities may generate false positive *P. vivax* pLDH lines. Maastricht Medical Students' Research Conference 2010. *Poster presentation*

Scientific awards

Both prizes were awarded for the research 'Malaria Rapid Diagnostic tests (RDTs) in the Peruvian Amazon' which I performed during my medical studies in Iquitos, Peru.

- Student Prize 2011 for excellent research, Maastricht University
- Prof. Chris Gips student prize 2012 for scientifically most talented medical student in The Netherlands. www.professorchrisgipsfonds.nl

Grants and scholarships

- Steunfonds 'Marguerite-Marie Delacroix': grant covering salary for a 2-year period for working on my PhD project 'Malaria in Children: accuracy and quality of Rapid Diagnostic Tests'
- FWO travel grant for a long stay abroad: research stay in Nanoro, Burkina Faso, for the study severe malaria & invasive bacterial disease and the study on invasive salmonellosis.
- Junior Mobility program KU Leuven: this program is an extra stimulus for outgoing mobility of junior KU Leuven researchers. Awarded for research in Burkina Faso.
- McKinsey & Company: grant for students for a project that will increase personal development. Grant received for research project 'severe malaria & invasive bacterial diseases in children' Burkina Faso. www.mckinsey.com/beurs